

**The Development and Validation of a Murine
Model for Studying the Role of Histamine
Receptors in Acute and Chronic Itch**

By

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Declaration

I declare that this thesis was composed entirely by myself, and the work on which it is based is my own with the following exceptions noted below and acknowledged in the text:

The automated detector of scratching was designed and constructed by Dr. Harry Brash, with the assistance of Derek Christie (Medical Physics, University of Edinburgh).

Honours research project students (Tamsin Blyth and Karen Sanders) provided assistance in gaining data for the following; control data for figure 3.1, 5-HT antagonist data for figures 6.2 and 6.3, and antagonist data for papain/SLIGRL for figures 6.6 and 6.7

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Abstract

Itch (pruritus) is an unpleasant sensation of the skin, which evokes the desire to scratch. The condition commonly presents in clinical practice, as a symptom of systemic disease and various skin disorders, such as atopic dermatitis. Itch has tended to be closely associated with pain, and older evidence suggests that the sensations involve the same sensory nerves, firing at different frequencies. Partly because of this, and the subjective cognitive nature of the condition, itch has been relatively ignored as a research area and there is a general lack of effective animal models for studying itch. This has restricted detailed studies into putative mediators of itch and their mechanism(s) of action.

The present studies were undertaken to develop and validate acute and chronic models of itch in mice, based on the combined use of behavioural tests in awake mice and *in vivo* electrophysiological recordings from itch afferents in anaesthetized animals. The hypothesis was that scratching behaviour can be evoked in mice using intradermal injections of pruritogenic drugs and that this can be measured automatically and objectively to provide a reliable indicator of itch. A further hypothesis was that electrophysiological recordings made *in vivo* from murine cutaneous sensory nerves can be used to distinguish between pruritogens and algogens.

The model of itch that was developed is based on injection of histamine into the back of the mouse neck to evoke scratching of the area by the hind paws. Histamine is a pruritogen in both humans and mice, although the lack of effectiveness of traditional H₁-receptor antihistamines in treating all clinical itch disorders suggests other mediators are also responsible for pruritus. The studies demonstrated that scratching in mice can be induced using histamine and other pruritogens (e.g. trypsin and 5-HT) in a reproducible dose dependent manner. Scratching was established as a response to itch-provoking agents, but not to painful stimuli. A novel mechanism of histamine evoked scratching involving H₄ receptors was discovered. Chronic itching in response to topical application of dinitrochlorobenzene was also established. A robust automated method for the detection and measurement of scratching in mice

was developed, which considerably enhances accuracy and reduces the time taken, in comparison with manual observation of scratching.

In vivo electrophysiological recordings showed that pruritogens evoke a pattern of response in cutaneous nerves distinct in nature from that evoked by algogenic stimuli. However, nerves responded to both stimuli, suggesting that in mice, there are probably no independent 'pruritoceptors', unlike the situation in man.

In summary, scratching in mice can be recorded automatically and used as a reproducible quantitative measure of itch. This model can be used for further studies on putative mediators of itch to establish their mechanism of action. Knowledge from such studies should provide understanding of the sensation of itch in man, and should facilitate the development of novel therapies specific for pruritus.

Abbreviations

ANOVA	analysis of variance
c	centi (10^{-2})
°C	degree centigrade
ED ₅₀	dose producing 50% of the maximum dose
FCA	Freunds Complete Adjuvant
g	gram
Hz	hertz
H _{1/2/3/4}	histamine receptor subtype 1,2,3 and 4
HCl	hydrochloric acid
HTMT	histamine-trifluoromethyl-toluidine
i.d.	intra-dermal
i.p.	intra-peritoneal
k	kilo (10^3)
l	litre
μ (prefix)	micro (10^{-6})
m (prefix)	milli (10^{-3})
min	minute
mol	mole
n	number
n (prefix)	nano (10^{-9})
PBS	phosphate buffered saline
P	probability
PAR	protease activated receptor
%	percent
s	second
sem	standard error of the mean
V	volt
w/v	weight per volume

Associated Publications

- BELL, J.K., MCQUEEN, D.S. & REES, J.L. (2004). Involvement of histamine H₄ and H₁ receptors in scratching induced by histamine receptor agonists in BalbC mice. *Br J Pharmacol*, **142**, 374-80.
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Chapter 1

General Introduction

Itch, clinically known as pruritus, is one of the most common symptoms presented clinically to dermatologists. In recent times, itch has become commonly defined as an unpleasant sensation of the skin that provokes the desire to scratch. Itch is a subjective sensation, and in the main is measured quantitatively by the amount of scratching that is elicited.

The scratching reflex operates when the skin is presented with an itch inducing stimulus and is likely to occur for one of two main reasons; firstly, the scratching of the area is likely to remove any irritant present on the surface of the skin causing the itch (and potentially skin damage/infection/pain); secondly, it is suggested that the mechanical activation of sensory nerves in the skin (e.g. touch or pain nerves) can ‘shut-off’ the itch sensation, through a neural gating system.

In the past, most studies into itch have been based on humans; clearly the fact that they can confirm the sensation being perceived provides more convincing evidence that itch is being studied. The same can’t be said of studies using animal models; it can only be assumed that the scratching response is due to a sensation the animal feels as similar to that humans describe as ‘itchy’.

Despite possible inadequacies compared to a human model, it is scientifically beneficial to have a reliable animal model. An animal model provides us with the background to investigate a wider range of potential pruritic (itch inducing) and anti-pruritic (itch relieving) drugs, in a much more intensive and also ethical manner. The general aim of the studies in this thesis is to attempt to create and validate a reliable and reproducible animal model of itch, using scratching as the main indicator of the sensation.

1.1 Neural Mechanisms of Itch Perception

During the last hundred years or so there have been great developments in our understanding of itching and scratching. Clearly the sensation of itch was present

long before this time, and although commented on by people as long ago as Socrates, there was no physiological understanding of the condition. Itch is a sensation; therefore it is the brain's interpretation of the environment. This clearly relies upon there being higher centres involved with the sensation of itch. The focus of the studies in this thesis is on the peripheral sensation of itch and not central pathways, however, in this section I will discuss briefly what is known of both peripheral and central neural pathways in order to provide a more comprehensive picture of how the sensation is elicited and interpreted.

The sensation of itch can be evoked either through a peripheral mechanism, known as 'pruritoceptive' itch, or through a central mechanism, known as 'neurogenic' itch. The early studies investigated pruritoceptive itch but in recent years there has also been an increase what is known of which areas of the brain contribute to the sensation.

'Itch Receptors'

In the 1950's the first systematic study to investigate the presence of 'itch receptors', or pruritoceptors, in skin, was reported. The work of Shelley and Arthur was based on the introduction of cowhage (*Mucuna pruriens*, the active ingredient of itching powder) spicules into the skin; they found that when these spicules were placed at the dermal-epidermal boundary that the sensation of itch was elicited (Shelley & Arthur, 1957). This work was the first to show where the sensation of itch is detected; the areas around the site of itch perception were termed 'itch spots'.

'Itchy skin'

'Itchy skin' was a phrase first used by Bickford in the 1930's and refers to a characteristic of itch that is very similar to that of hyperalgesia and its relation to pain (Bickford, 1938). The phrase itchy skin has now been replaced by the more technical term allodynia and describes a situation in which the sensitivity of skin adjacent to that which has recently been exposed to an itchy stimulus, becomes more sensitive. That is to say when itch is induced in one area of skin, the skin around that

area is more susceptible to the sensation of itch when rubbed. This phenomenon was shown to last in the surrounding skin up to 15 minutes after the initial itch stimulus, approximately twice as long as the duration directly caused by the itch stimulus itself. Bickford described the presence of itchy skin as being a result of a 'local nervous pathway'. This local nervous pathway can be used to explain the findings of Lewis in the 1920's. Lewis found that administration, into the skin, of what we now know to be histamine induces itching. He also identified the triple response to histamine (Lewis, 1927); itch, wheal and flare. This triple response elicits swelling and reddening of the area as well as the sensation of itch. Effectively the reaction is an immune associated response; the release of neuropeptides from nerves around the area, and the activation of local immune cells such as T-cells and mast cells, many of which have the potential to activate other nearby nerves.

The 'anti-pruritic state'

A notable characteristic of itch was described by Bickford and further investigated by Graham in the 1950's. The anti-pruritic state was used to define a situation where induction of pain in an area of skin, prevented the induction of itch in that area (Bickford, 1938; Graham *et al.*, 1951). At that time the consensus of opinion was that itch and pain must therefore be different intensities of the same sensation; a small noxious stimulus would elicit itch and if increased, pain would be sensed. Rothman presented work suggesting tickle was the least intense sensation and that this progressed into itch and then pain (Rothman, 1941).

Nociceptive nerve fibres and itch nerve fibres

With time, the opinion that the afferent nerve pathways for pain and itch were the same became less popular. Support that the neural pathways for pain and itch are distinct came with the development of the gate-control theory (Melzack & Wall, 1965). This theory describes how the sensations of pain and itch can use separate pathways, yet still interact centrally. It was proposed that activation of fast conducting, large diameter axon, myelinated A- β nerve fibres, which are usually activated by touch and pressure, acts at the level of the spinal cord to reduce activity

in the slower conducting, thin diameter axon, myelinated A- δ and unmyelinated C-fibres, believed to be associated with noxious stimulation and also possibly itch. The debate as to whether pain and itch sensations were transmitted using separate or the same nerve fibres continued, and even into the 1990's some authors concluded the two sensations used the same pathway (Greaves, 1997).

McMahon and Koltzenburg provided a summary of the four different possibilities of the neural transduction of the pain and itch sensations (McMahon & Koltzenburg, 1992). The four possibilities that were presented were the specificity theory, intensity theory, pattern theory and the selectivity theory and are discussed briefly below.

The *specificity theory* is the least complicated and suggests that itch and pain are relayed via completely independent nerves. Pruritogenic stimuli activate 'itch nerves' and painful stimuli activate 'pain nerves'. This theory was commonly discounted because of the lack of evidence for the existence of nerve fibres that respond only to pruritogenic stimuli. It had been shown in nerve recordings from C-fibres in humans that both itch and pain-inducing drugs activated the same nerve fibres (Handwerker *et al.*, 1991). It had also been shown that micro-stimulation of C-fibres was not conclusively able to elicit an itch rather than a pain sensation in human subjects, although it was possible in a limited number of cases (Torebjork & Ochoa, 1980).

The *intensity theory* is that which was most commonly held during the middle of the 20th century and is based on work presented by Rothman, as discussed above. In practice, however, this theory would appear flawed. The administration of opiates as an epidural reduces pain, however, itching is a common side affect (Ballantyne *et al.*, 1988; Harrison *et al.*, 1988). This is very difficult to explain if the two sensations are carried through the same nerve fibres. Also micro-stimulation of C-fibres at different frequencies does not elicit separate sensations of itch and pain (Ochoa & Torebjork, 1983).

The *pattern theory* proposes that by activating the same nerve in a separate way, the brain can identify between two stimuli, or, that evoked by activating a subset of

nerves in an area in a different way, the brain can differentiate between stimuli. This theory seems ideal for explaining the similarities between itch and pain, but has no conclusive evidence to support it. The first possibility can be discounted as it has been shown in C-fibre nerve recordings in humans that changing the pattern of activation does not change the sensation perceived by the subject (Torebjork & Ochoa, 1980). The second option is that by activating a different sub-population of nerves in an area, you can elicit a separate sensation. Therefore, by stimulating fewer nerves in an area of skin you may elicit itch, and by activating a few more this may transfer into pain (Handwerker, 1992). This would be almost impossible to achieve experimentally without knowing the 'code' and as yet no evidence has been found to suggest it occurs.

The final theory is the *selectivity theory* that suggests that there are no specific itch nerves, but a subset of pain nerves which also responds to an itch stimulus (Handwerker *et al.*, 1991). When activated these nerves could be responding to an itch or pain stimulus, what is perceived is dependent on central 'itch processing'. If no other 'pain nerves' are being activated, the sensation is perceived as itch, if other pain nerves are activated the sensation perceived is pain, therefore, pain and itch cannot co-exist. The theory also goes on to describe that the spinal processing is opioid mediated, thus explaining why epidural, as described above, can relieve pain but enhance itch, it suggests disruption of the feedback system allows the itch sensing fibres to fire without being inhibited by the firing of other pain fibres.

Despite the selectivity theory seeming plausible, the more recent evidence in this field has given weight to the specificity theory. It has been shown that there are itch-specific C-fibres in human skin, which respond selectively to histamine (Schmelz *et al.*, 1997) that were previously believed not to exist. It has also been shown that in cats there is a population of spinothalamic tract neurons (secondary neurons) that respond selectively to histamine (Andrew & Craig, 2001). There is now increasing evidence to support a specificity theory of itch, over the others discussed earlier.

Central Pathways of Itch

Various authors have presented evidence to suggest that the itch sensation is carried via C-fibres (Fjellner, 1981; Graham *et al.*, 1951; Handwerker *et al.*, 1991; Ochoa & Torebjork, 1983; Schmelz *et al.*, 1997; Shelley & Arthur, 1957; Torebjork & Ochoa, 1980), the slowest conducting ($<2\text{ms}^{-1}$) unmyelinated nerve fibres. These nerve fibres pass to the central nervous system via the dorsal root to the dorsal horn of the spinal cord. Here the primary afferent nerves synapse to secondary nerve fibres that pass to the brain in the contralateral spinothalamic tract (Greaves, 1997). It has recently been shown that these second order nerves in the spinal cord are selectively responsive to histamine and are proposed to be central 'itch nerves' (Andrew & Craig, 2001). Support for this being the neural pathway for itch is provided by the evidence that the itch sensation is abolished by spinothalamic tract cordotomy (Nathan, 1990). These second order nerves pass to the thalamus (Kam & Tan, 1996).

Little evidence exists to describe the pathway for the itch sensation in the brain; however, recent evidence using positron emission topography (PET) provides a clue as to which brain areas become activated due to an itch stimulus. These studies have shown stimulation of various areas of the cortex during the sensation of itch. The areas of the brain identified as being stimulated include; the cingulate cortex, related to the identification of a noxious stimulus; the premotor and supplementary motor areas, related to the planning of motor activities and the inferior parietal lobule, related to the initiation of movement (Hsieh *et al.*, 1994; Savin, 1998). Together this evidence suggests that there is not one single itch centre and also that the central activation due to an itch stimulus appears to be related to initiation of a scratch response.

1.2 Clinical Pruritic Conditions

Pruritus commonly presents as a symptom not only of conditions of the skin, but can also be a secondary symptom of a variety of systemic disorders. It is important to remember that it is always preferential to treat the underlying condition causing the

itch and not to focus solely on the sensation of itch itself. Having said this, over recent years the pharmaceutical industry has invested in developing itch-relieving products, primarily designed to be over-the-counter 'lifestyle' drugs.

In this section I will discuss briefly some of the various disorders for which pruritus is commonly the presenting symptom. In many cases the pathophysiology of the itch in these conditions is poorly understood and so it may be crude to define many of these disorders so generally. However, I have grouped the itch as being due to one of three underlying reasons; firstly, due to an underlying systemic disease, secondly, caused by a disorder of the skin, and finally, itch presenting with apparently no underlying clinical disorder.

Itch Associated with Systemic Disease

Chronic Renal Failure

Chronic renal failure (uraemia) at onset does not generally produce itch, however, it seems to be the treatment that provokes the sensation. Itch can occur prior to, during or after dialysis. This makes determining the cause difficult. Earlier evidence showed up to 85% of patients suffered from itch (Stahle-Backdahl, 1992; Young *et al.*, 1973), whereas recent evidence would suggest that this level may now be as low as 25%, this is most likely to be due to improvements and developments in the dialysis treatment provided to these patients (Pauli-Magnus *et al.*, 2000).

Although the dialysis process clearly plays a part in both relieving and causing itch in uraemic patients, due to accumulating or removing pruritogens, there are clearly other factors involved. There has been a wide range of suggestions as to the cause of the itch but as yet none have been convincingly shown to be responsible for the itch felt by uraemic patients. It is suggested that associated secondary hyperparathyroidism can induce itch (Massry *et al.*, 1968), and is treatable by parathyroidectomy (Hampers *et al.*, 1968). There is also evidence that the number of mast cells present in the skin of uraemic patients is higher than healthy subjects (Leong *et al.*, 1994). The plasma level of histamine in patients with uraemic itch is elevated, but the level of histamine would appear not to correlate with itch severity (Mettang *et al.*, 1990).

The skin of uraemic patients is drier than that of non-uraemic patients (Young *et al.*, 1973) and this may also cause itch, however, changes of the skin such as those discussed could well be a result of the itch and not the cause itself. More recently a role for opioid receptors has been proposed due to the observation of a change in the relative expression of μ - and κ - receptors on lymphocytes (Togashi *et al.*, 2002). The opioid receptor antagonist naltrexone has provided mixed evidence of itch relief (Pauli-Magnus *et al.*, 2000; Peer *et al.*, 1996) and it may be that these opioid effects are more significant in a central pathway and not peripherally.

Hepatic Cholestasis

Disorders of the liver are often associated with a severe and debilitating itch, most commonly seen in obstructive jaundice. The subsequent elevation in bile salt levels was seen as the explanation for the itch, although this may in part be true, it does not appear to be the sole reason. Elevated plasma bile salt levels are reported to cause itching (Ghent *et al.*, 1977; Kirby *et al.*, 1974), however, there appears to be no direct correlation between the level of plasma bile level and the degree of itch reported by the patient (Jones & Bergasa, 1990).

Recent evidence implicates a role for endogenous opioids being involved in the itch caused by cholestasis. Changes in the opioidergic tone is said to affect the itch centres of the brain during liver disease (Bergasa & Jones, 1995; Jones & Bergasa, 1999). This suggestion is supported by the evidence that opioid antagonists, such as naloxone, are effective at relieving cholestatic pruritus (Bergasa, 1995).

Haematological

Conditions of the blood are often associated with itch. 50% of patients with polycythaemia vera present with itch and in patients with Hodgkin's lymphoma the figure is as high as 30%, other conditions include leukaemia and T-cell lymphoma (Botero, 1978; Rajka, 1966; Winkelman, 1964). The causes of the itch are unknown, although polycythaemia vera is often associated with elevated histamine levels (Archer *et al.*, 1988), but treatment can only be focussed on the underlying condition.

Iron deficiency can present clinically as pruritus in patients with normal skin (Lewiecki & Rahman, 1976), however, studies have revealed it would appear not to be directly related to the iron levels in the blood, but another, as yet unknown factor (Tucker *et al.*, 1987).

Endocrine

The most common endocrine disorder to be linked with itch is diabetes mellitus, however, this is simply not true (Greaves, 1993). It was reported in a single article in 1927 and has been presented incorrectly since that time (Greenwood, 1927), in fact only 3% of diabetes patients complained of itch and the degree did not correlate with the severity of diabetes.

Other endocrine disorders do, however, present as pruritus. Hyperthyroidism has been shown to cause itch (Carvati *et al.*, 1969; Rothfield, 1968) and this may, at least in part, be due to vasodilatation and increased blood flow to the skin (Fruhstorfer *et al.*, 1986). Postmenopausal women also commonly present with itch associated with hot flushes, thought to be reliant upon plasma levels of gonadotrophins (Greaves, 1993).

Malignant Tumours

The presence of a malignant tumour can present as a generalized itch and this can be months or even years prior to the discovery of the tumour itself (Cormia, 1965) (Andreev, 1978; Lober, 1993). Generalized itch normally occurs due to the presence of a visceral tumour, however, a localised itch can also occur, most commonly due to the presence of a tumour in the brain (Andreev & Petkov, 1975).

HIV/AIDS

Itch occurs in patients with HIV and AIDS, both with and without associated skin abnormalities (Shapiro *et al.*, 1987). In many cases the itch is due to a wide range of secondary conditions including skin infections or other systemic disease. However, some patients complain of itch without any obvious skin or systemic disorder

(Hoover & Lang, 1991). Recently it has been shown that HIV/AIDS patients have an increased incidence of a condition known as eosinophilic folliculitis, this is a pruritic condition in which papules or plaques form on the skin and appears to be linked with increased IgE serum levels (Rodwell & Berger, 2000).

Neuropathic

It is reported that abnormalities of the nervous system can lead to itch. One condition in which this is common is multiple sclerosis (Yamamoto *et al.*, 1981). It is suggested that in instances such as this the cause of the itch is due to abnormal synaptic transmission rather than damage to the nerve axon through demyelination (Sandyk, 1994).

Itch Associated with Disorders of the Skin

Atopic Eczema

Eczema is a condition associated with drying and lichenification of the skin and is intensely itchy in all patients. Changes in the skin of eczema patients provides an indication as to the cause of the itch although treatment for the condition focuses on treating the dryness of the skin using emollients and reducing inflammation with corticosteroidal cream. Changes in the skin have been shown to include an increase in the number of mast cells (Mihm *et al.*, 1976). It is also believed there may a prominent role for a central as well as peripheral mechanism in the itch of eczema patients. This suggestion is based on the finding that low sedation antihistamines are less effective than the older sedatory antihistamines (Krause & Shuster, 1983). The effectiveness of opioid antagonists in relieving the itch in some patients also supports the suggestion that a central role may be important in the itch of atopic eczema (Monroe, 1989).

Psoriasis

Up to 80% of patients with psoriasis suffer from itch (Krueger *et al.*, 2001), however, the itch is not necessarily localised to the affected areas of skin. Inflammation of the skin occurs and this is most likely to be the cause of the itch, but there is also an

increase in sensory nerve innervation to the skin that could explain the pruritus (Naukkarinen *et al.*, 1989).

Urticaria

Urticaria is the itch associated with a release of histamine and other endogenous compounds from mast cells as an immune response, such as insect sting or bite. Urticaria is one of the most easily treated forms of pruritus and can be alleviated using a traditional antihistamine. The itch associated with urticaria is localised to the affected area of skin.

Superficial Parasitosis

When skin is physically damaged due to a parasitic infection it can often present as a feeling of itch. The classic example is that of scabies, the associated inflammatory response is the most likely cause of the itch although which mediator is responsible is unknown.

Miscellaneous Itch Conditions

Aquagenic Pruritus

Aquagenic pruritus is a disorder characterised by intense itch with no apparent changes in skin condition. The itch appears on any area of skin wetted by water, of any temperature, and lasts for up to one hour (Greaves *et al.*, 1981). It has been reported that anywhere up to 3.3% of the population may suffer from such a condition (Potasman *et al.*, 1990), however there are no reported systemic effects.

Senile Pruritus

It is reported that up to 50% of people aged 70 or above suffer from this condition (Twycross *et al.*, 2003). Often there may be underlying causes of the itch, such as dryness of the skin, lack of moisture of the skin, or an underlying disorder, however, true senile pruritus presents with no apparent skin abnormalities.

1.3 Mediators of Itch

Evidence as to which endogenous compounds evoke the sensation of itch have been derived from both experimentally-induced itch in healthy subjects as well as studies into patients with clinical disorders of which itch is a symptom. In the following section I will focus on what is known of the various mediators of itch in humans. In many cases the evidence to date may appear incomplete and hypothetical and the exact mechanism of how the compound evokes itch is not clear. In some cases the examples used are not endogenous to human skin, however, the tools used provide an indication as to a possible *in vivo* action of related compounds.

Histamine

Some of the earliest research into itch found the compound now known as histamine to be present in increased quantities in the skin of 'itchy patients'. The substance was at that time referred to as 'H-substances'. It was shown to induce itch when injected superficially, but to cause a burning pain when administered less superficially (Lewis, 1927). Since these early times histamine has been widely regarded as the stereotypic itch inducer in humans and normally forms the benchmark against which experiments are performed, in both human and animal models. Work in the 1960's by Keele and Armstrong was the first comprehensive research of experimentally induced itch using histamine in humans. This work provided the first details of dose required to evoke itch, volume in which the histamine should be administered to maximise the itch response and how the histamine should be administered (intradermal injection was found to be the most effective) (Keele & Armstrong, 1964).

Endogenous histamine is stored in mast cells located in the dermis, activation of the mast cells (for example as part of an inflammatory response) causes degranulation and histamine release. Histamine acts upon one of four receptors; H₁, H₂, H₃ and H₄ (Leurs *et al.*, 2001). The role histamine H₁ and H₂ receptors in itch have been well characterised (see below), however, until now there has been no evidence in the literature to support a role for involvement of either the H₃ or H₄ receptor in itch.

Chapter 5 of this thesis investigates the role of the different histamine receptors in itch in more detail. Histamine induced itch has been shown to operate through the histamine H₁, but not the histamine H₂ receptor (Davies & Greaves, 1980; Hagermark *et al.*, 1979). However, although traditional antihistamines (H₁ receptor antagonists) can reduce experimentally induced itch they are not effective in all itch conditions, most typically, the itch associated with atopic eczema (Berth-Jones & Graham-Brown, 1989; Wahlgren *et al.*, 1990). Despite the lack of strong evidence to support a direct role of the histamine H₂ receptor in itch, H₂ receptor antagonists are used clinically in some conditions. A combined H₁ and H₂ receptor antagonist therapy is used in the treatment of urticaria (Monroe *et al.*, 1981). The effectiveness of the combined drugs may be due to the possible role the H₂ receptor plays in the vasodilation of the blood vessels in the skin, more than its direct effect on relieving itch (Marks & Greaves, 1977).

Although recognised as a pruritogen, histamine does not appear to play an integral role in many clinical itch conditions. It has been suggested that traditional antihistamines may have central sedatory actions in itch relief, more important than direct peripheral actions, at least in conditions such as eczema, where the major mediator is not believed to be histamine (Krause & Shuster, 1983; Savin *et al.*, 1986).

One final piece of evidence suggests that histamine is not a major mediator of itch in chronic skin conditions; repeated histamine administration results in a gradual loss of itch response, this is referred to as tachyphylaxis (Stahle-Backdahl *et al.*, 1988). If histamine induces tachyphylaxis, it is fair to assume that histamine could not induce chronic itch conditions, as the sensory nerves would be desensitised and no longer relay the itch sensation.

Despite the ambiguity of histamine as a major mediator of itch in clinical conditions, it has nevertheless been shown to play a role in mediating itch and is to this point the most widely investigated pruritogen and the most common target in therapeutic treatment.

Mast Cell Degranulation

As discussed previously, the effects of histamine *in vivo* occur through the release of histamine from mast cells. Various endogenous compounds induce this effect (see below) but one useful tool in this field is the compound 48/80. This drug when administered intradermally causes mast cell degranulation and elevated histamine levels, with an associated itch (Ruckwied *et al*, 2002; Fjellner & Hagermark, 1981). Compound 48/80 is of use in two ways; firstly it induces a mast cell-like itch, useful in evoking acute models of itch and secondly it can be used to degranulate mast cells prior to the administration of another pruritogen, providing an indication as to whether these other pruritogens evoke their effects through the activation of mast cells or through a separate mechanism.

Neuropeptides

Neuropeptides are a group of peptides synthesized and released by nerves. Among these compounds identified from peripheral nerves in the skin are substance P, vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), secretin, somatostatin, neurotensin, neurokinin A and neuropeptide Y. These peptides are released to act as neurotransmitters or modulators acting on nearby cells, such as mast cells (Hokfelt *et al.*, 1980).

The most widely studied neuropeptide with regards to the induction of itch is substance P. Substance P is synthesized in the dorsal root ganglion and transported to the periphery down the nerve axon, where it is co-localized with the other neuropeptides CGRP and VIP in the nerve terminals (Bloom *et al.*, 1988). Administration of substance P intradermally induces itch in healthy subjects (Ward *et al*, 2004; Fjellner & Hagermark, 1981; Hagermark *et al.*, 1978), it is suggested that this acts through the release of histamine from mast cells (Ebertz *et al.*, 1987; Jorizzo *et al.*, 1983) and is associated with the same triple response seen with a histamine response.

Many neuropeptides have yet to be studied as potential mediators of itch, however, secretin, neurotensin and VIP have been reported to be pruritogenics (Misery et al, 2003). The actions of these compounds have been shown to act through mast cells and subsequent histamine release as their actions are reduced by prior administration of the H₁ antagonist, mepyramine or the mast cell degranulator, compound 48/80 (Fjellner, 1981). Another of the neuropeptides, CGRP, has been shown to have no itch inducing properties (Brain & Williams, 1985). A later study confirmed this finding and also contradicted the earlier report that VIP was pruritogenic, by stating that neither compound could evoke itch in man (Greaves & Wall, 1996). Despite this evidence there is a reduction in the number of CGRP immunoreactive sensory nerves following phototherapy in humans, which is recognised clinically to be effective in reducing itch (Wallengren & Sundler, 2004). Whether CGRP is involved directly with the itch sensation will require further investigation.

Proteases/kinins

Proteases were first shown to play a role in mediating itch through work using spicules of the plant cowhage, *Mucuna pruriens*, inserted into the dermis of the skin (Shelley & Arthur, 1955). They reported that the active ingredient of the spicules was a protease enzyme, mucunain, based on evidence that spicules that had been boiled prior to being inserted into the dermis evoked no itch, due to denaturing of the enzyme.

A search for endogenous proteases, and the substrates from proteolysis, was undertaken by Monash and Woessner. They presented evidence for itch evoking properties of ficin, papain, pancreatin and chymotrypsin (Monash & Woessner, 1958). The mechanism by which these proteases induce itch would appear to be different. The itch evoked by trypsin and chymase is associated with a triple response similar to that induced by histamine and the itch is relieved by administration of antihistamines (Hagermark *et al.*, 1972). However, the itch evoked by two other proteases, papain and kallikrein, was not reduced by antihistamines and there was no triple response (Hagermark, 1974).

Bradykinin is a peptide substrate formed by the breakdown of kininogen by kallikrein that has been studied as a possible mediator of itch (Stander et al, 2003). It has been shown that intradermal administration of bradykinin produces a sensation of pain and not itch (Greaves & Shuster, 1967), however, it has also been reported that bradykinin can potentiate the itch evoked by histamine (Schmelz et al, 2003; Hagermark, 1974). The mechanism of potentiation is still unknown, however, and has the potential to provide an interesting insight into the mechanisms of itch in clinical disease.

The evidence suggests that proteases can evoke itch through both a histamine and a non-histamine pathway. Proteases also act directly on protease-activated receptors (PARs). There are four PARs discovered to date, PAR-1, PAR-2, PAR-3 and PAR-4 (Macfarlane *et al.*, 2001; Vergnolle *et al.*, 2001b). They are implicated as playing a role in inflammation responses. One of these receptors, PAR-2, is also suggested to be involved in mediating the sensation of itch (Steinhoff *et al.*, 2003). The receptor is found on C-fibre nerve terminals and is activated by tryptase released from mast cells (Steinhoff *et al.*, 2000; Compton *et al.*, 2000). As yet the exact functional role of PARs is unclear, however, further investigation may reveal they are involved in itch.

Opioids

The endogenous opioids are a family of peptides with morphine-like activity referred to as endorphins or enkephalins (Hughes *et al.*, 1975). They are a specific group of neuropeptides located in both central and peripheral nerves, commonly recognised to play a role in the perception of pain (Beaumont & Hughes, 1979). Due to the wide distribution of the opioids throughout the nervous system it is proposed that they play a role in mediating itch both centrally and also peripherally.

Peripherally administered morphine results in mast cell degranulation and itching, characteristic of histamine release, i.e. the triple response of itch, weal and flare (Feldberg & Paton, 1951). Morphine and beta-endorphin, injected intradermally, also potentiates the itch response induced by histamine injection (Fjellner & Hagermark, 1982), however the potentiation effect is not eliminated by prior mast cell

degranulation using compound 48/80 (Fjellner & Hagermark, 1982). The potentiation of histamine induced itch by morphine and beta-endorphin is also unaffected by the morphine antagonist, naloxone (Fjellner & Hagermark, 1984). It would therefore appear that opiates are capable of potentiating itch through a pathway that is non-opiate and non-histaminergic, as yet the mechanism of this potentiation is still unknown.

There would also appear to be role for opiates in mediating itch through central mechanisms. Morphine has been shown to cause itch in up to half of the patients receiving an epidural injection (Ballantyne *et al.*, 1988; Harrison *et al.*, 1988). The itchy areas of skin are not affected by the triple response associated with histamine and so the effect is probably not mast cell related, but may be the effect of potentiating the pruritogenic effect of another compound (Scott & Fischer, 1982). The itch inducing effects of spinally administered opiates are removed by the opiate antagonist naloxone (Vedrenne *et al.*, 1991), however, the evidence to date does not conclusively suggest there is a role for naloxone in relieving clinical itch conditions (Bernstein & Swift, 1979; Summerfield, 1981). There are reports to suggest another opioid antagonist, nalmefene, is effective at reducing the itch associated with liver disease (Thornton & Losowsky, 1988), and that of atopic eczema (Monroe, 1989). Interestingly, more recent reports have indicated that the itch induced by spinally administered opiates is reduced by the 5-HT₃ receptor antagonist, ondansetron (Borgeat & Stirnemann, 1999; Kyriakides *et al.*, 1999). This evidence provides the first insight that the itch inducing properties of opiates may involve the 5-HT pathway.

5-Hydroxytryptamine (5-HT, Serotonin)

5-HT evokes itch when injected intradermally into human skin. It is considered a weak pruritogen and the degree of itch, even at high concentrations is not considered as strong as that induced by histamine (Fjellner & Hagermark, 1979). However, it has been shown to induce a more intense itch when co-injected with prostaglandin E₂, PGE₂ (Fjellner & Hagermark, 1979). 5-HT is not stored in human mast cells, although it is in the mast cells of rodents, and the primary source of 5-HT in the skin

is from platelets (thrombocytes), from where it is released during platelet aggregation (Hagermark, 1992). Although 5-HT is not stored in mast cells it has been reported that 5-HT induced itch is mast cell dependent, operating through the histamine pathway, with no itch occurring following prior mast cell degranulation with compound 48/80 (Weisshaar *et al.*, 1999). In polycythaemia vera, it is believed that the itch is caused by the simultaneous release of 5-HT and prostaglandins, this has been confirmed by the finding that 5-HT antagonists are effective at relieving the itch of this condition (Tefferi & Fonseca, 2002; Fitzsimons *et al.*, 1981).

The 5-HT₃ receptor antagonist, ondansetron, is effective at relieving the itch induced by spinally administered opiates (see above). The same drug is used in the treatment of itch in patients with cholestasis (Schworer & Ramadori, 1993) and itch during pregnancy (Schumann & Hudcova, 2004), however, as there are no reports of the 5-HT₃ receptor being expressed in the human skin it is proposed that these effects are centrally operating (Jones & Bergasa, 1999). This hypothesis fits with the evidence that suggests the opiates also operate at a central level.

Platelet activating factor (PAF)

PAF is a phospholipid, released by proinflammatory cells due to the activity of phospholipase A₂. It has been shown to be pruritic in humans, with its activity being reduced by antihistamines or prior mast cell degranulation using compound 48/80 (Kato *et al.*, 2003; Thomson *et al.*, 2002; Archer *et al.*, 1988; Fjellner & Hagermark, 1985).

Eicosanoids

Eicosanoid is the collective term for the derivatives of arachadonic acid. These substances are commonly associated with inflamed skin and include the prostaglandins and leukotrienes and are released from a host of proinflammatory cells (Greaves *et al.*, 1971; Voorhees, 1983). Prostaglandins E₁, E₂ and H₂ are reported as having little or no pruritic activity when injected intradermally into human skin, i.e. itch is not reported in all subjects and the itch sensation is described

as weak (Greaves & McDonald-Gibson, 1973; Hagermark & Strandberg, 1977). However, it has been reported that these prostaglandins act to lower the threshold itch response to a subsequent injection of histamine (Neisius et al, 2002; Hagermark & Strandberg, 1977). This lowering of threshold by prostaglandins is also seen in relation to pain responses (Ferreira, 1972). Prostaglandin E₁ also lowers the threshold response to intradermal injection of the protease papain (Lovell *et al.*, 1976). Prostaglandin D₂ does not lower the threshold response to histamine (Maurice *et al.*, 1987). It would appear the role of the prostaglandins is modulatory and not as primary mediators. This suggestion is supported by the evidence that aspirin, which blocks the activity of the enzyme cyclooxygenase in the prostaglandin synthesis pathway, and other non-steroidal anti-inflammatory drugs (NSAIDs) have no effect in relieving clinical itch conditions (Daly & Shuster, 1986; Hagermark, 1973), indeed aspirin appears to make skin more sensitive to histamine induced itch (Hagermark, 1973). Recently this finding has been disputed with evidence suggesting topically applied aspirin can act to reduce histamine induced itch (Yosipovitch *et al.*, 1997).

The other major member of the eicosanoid family, the leukotrienes, have yet to be investigated in the same depth as the prostaglandins in this field. Recent evidence points to an antipruritic effect of leukotrine B₄ (Storms, 2003), however, the evidence to date suggests they play no role in directly mediating itch in human skin because intradermal injections of leukotrienes B₄, C₄ and D₄ did not cause itch (Camp *et al.*, 1983).

Cytokines

Cytokines are a family of proteins produced by the action of antigens on a variety of immune response cells, most commonly lymphocytes, but also keratinocytes, fibroblasts and mast cells (Greaves & Wall, 1996; Hagermark, 1992). There are accumulations of cytokines in many clinical pruritic conditions, such as eczema, found in the epidermis and upper dermis (Katayama et al, 2001; Hagermark, 1992). Research into itch has so far focussed on two cytokines, interleukin-1 and interleukin-2. Interleukin-1 does not induce itch when injected intradermally (Dowd

et al., 1988). However, interleukin-1 does appear to play a role in the mediation of itch associated with carcinoma (Chi *et al.*, 2001; Woodlock *et al.*, 1999) and HIV (Breuer-McHam *et al.*, 2000). Administration of interleukin-2 either intravenously (Gaspari *et al.*, 1987) or intradermally induces itch (Darsow *et al.*, 1997; Wahlgren *et al.*, 1995). The itch inducing properties of interleukin-2 is present in the skin of healthy subjects and patients suffering atopic eczema (Wahlgren, 1995). A possible role for interleukin-2 in clinical itch conditions is supported by the evidence that cyclosporin A, which reduces interleukin-2 production from lymphocytes, reduces the itch associated with eczema (Hagermark & Wahlgren, 1992; Wahlgren, 1995). A role has also recently been identified for IL-18 as a pruritogenics compound in a spontaneous itch similar to that of atopic dermatitis (Konishi *et al.*, 2003). Another cytokine implicated by recent research is interferon gamma, a compound released by T-cells, which are immune response cells localised in the skin, this compound may play a role in the spontaneous itch of transgenic mice (Ny & Egelrud, 2003). To date there have been no investigations into the itch inducing properties of other cytokines.

Acetylcholine (ACh)

Acetylcholine is commonly recognised as a neurotransmitter in peripheral nerves, however, is not only produced in nervous tissue but also synthesized and stored in keratinocytes located in the skin (Grando *et al.*, 1993) and may have a role in the elicitation of itch (Heyer *et al.*, 2002). Levels of acetylcholine have been shown to be elevated in the skin of patients suffering from atopic dermatitis compared to healthy subjects (Reinheimer *et al.*, 1998). Interestingly, intradermal injection of acetylcholine into the skin of patients with dermatitis induces a sensation of itch, whereas in healthy subjects the perceived sensation is pain (Heyer *et al.*, 1997; Heyer & Hornstein, 1999). Conversely it has been found that patients with dermatitis are less sensitive than healthy subjects to intradermal injections of histamine (Rukwied *et al.*, 2000). These findings raise the possibility that clinical itch conditions could be due to a change in sensitivity to acetylcholine. The mechanism of action of acetylcholine induced itch is not clear, however, it is apparently not through histamine release from mast cells as administration of histamine H₁ receptor antihistamines are ineffective at reducing the itch (Rukwied & Heyer, 1998).

Alternative pruritogens

It is important to remember that the list of possible mediators of itch mentioned in the previous sections is not comprehensive. The evidence presented in the literature to date has been discussed but it is clear that there are a great number of potential mediators and associated receptors still to be investigated. In particular, the eicosanoids and cytokines have yet to be investigated in detail, with many compounds in these groups not studied with regards to itch. The major problem investigators have is that any endogenous compound present in the skin is a potential candidate, whether it directly activates peripheral nerves or acts via an indirect mechanism, such as mast cells and subsequent histamine release. The field is further complicated by the interactions between compounds leading to sensitisation of peripheral nerves to other pruritogens or potentiation of response due to interaction between compounds, such as that seen by some of the prostaglandins. Only further research will further clarify these questions.

1.4 Animal Studies

In this section the evidence for different mediators of itch, and the mechanisms by which they are believed to act, from animal studies, will be reviewed. The focus is mainly on studies in mice, as these formed the basis of the studies undertaken for this thesis. However, relevant findings from other species are also included.

There is a need to develop robust animal models of itch for a variety of reasons. Animal models are the most effective method of testing a range of compounds in a more intensive manner than is possible using small numbers of human volunteers or patients. Testing of novel compounds is also easier for ethical reasons prior to drugs receiving safety clearance for human investigation. There are also more mundane yet no less crucial benefits of animal models, such as economic cost.

The studies in this thesis are focussed on the development of a mouse model of itch. The main benefit of studies in mice as opposed to studies in other animals is the development in genetic manipulation. The recent growth in the field of transgenics has opened up new possibilities for discovering how endogenous compounds or receptors function in vivo. The ability to over or under-express specific targets gives us a new insight into the exact function of the target in question. In a field where we are examining a complex interaction between mediators and receptors, this may be the most effective method of establishing the specific role of these compounds, in the long term.

Different model types

The aim of any animal model is to provide an eventual end point which can be of direct relevance to human research and consequently to benefit the human population. With this in mind the most suitable models to develop and study would be animal skin conditions that are directly comparable to human clinical conditions. Unfortunately, such conditions do not occur naturally in mice. However, there are a couple of examples of mice with 'skin conditions'. An inbred strain of mouse, known as the NC mouse has been bred that would appear, to histological examination, to have an atopic dermatitis-like condition of the skin (Suto *et al.*, 1999; Vestergaard *et al.*, 2000). These mice show spontaneous scratching behaviour. The problem with a model such as this is that they are developed without knowing the exact cause of the condition and so still does not tell us the cause of the related human condition. Having said this, it is an animal model that can be investigated more rigorously than can the condition in human subjects and is potentially of great use in the future. Another such model exists that has used transgenic technology. Mice have been bred which express the cytokine, interleukin-4 in the epidermis (Chan *et al.*, 2001). These mice scratch spontaneously and have a skin condition similar to that of atopic dermatitis. The expression and itch inducing powers of interleukin-4 in the epidermis is not one currently presented in the literature. Again, the problem of this form of model is that we cannot be sure that the condition although similar to a clinical condition is identical in its pathophysiology. Despite their drawbacks, these disease models are of use for investigating skin abnormalities and it seems certain many

more such models will be developed in the coming years. More detail of chronically induced models of itch will be discussed in chapter 7 of this thesis.

Acutely induced scratching models

Human studies have not only been carried out in patients with skin disease but also using the administration of drugs into healthy subjects to evoke itch. The same can be attempted in animal studies. Problems with such models are based on the interpretation of the induction of itch. Animals clearly are unable to report a sensation of itch. In such studies scratching is assumed to equate to the sensation of itch. This assumption is not without its critics (McMahon & Koltzenburg, 1992), with some suggesting that pain elicits a scratch response in rodents. For example, scratching induced in arthritic rats is assumed to be due to chronic pain (De Castro-Costa *et al.*, 1987). Despite its critics, acutely induced scratching behaviour is the most common form of animal model of itch. It has been shown that in mice scratching is evoked by pruritogenic, compound 48/80 and substance P, not algogenic, capsaicin and formalin, drugs (Kuraishi *et al.*, 1995).

Another problem in developing murine models of scratching is reproducibility. If the model does not consistently present the same evidence it is of limited use. Such problems can arise due to variables not being controlled, most commonly these would be strain differences and age-related differences. It has been reported that different strains of mouse vary in their responses to different itch inducing drugs. For example the BalbC strain of mouse is significantly less responsive to histamine than the ICR strain, yet, both these commonly used strains respond similarly to another pruritogen, 5-HT (Inagaki *et al.*, 2001). It is also reported that there are strain related differences in the release of histamine from mast cells (Toda *et al.*, 1989). It is important these differences are appreciated before results interpreted and conclusions drawn. Significant differences can also exist between individual mice dependent on age. BalbC mice have been shown to have a variable number of mast cells between the ages of six and ten weeks (Hart *et al.*, 1999), therefore changes in scratching response over this time period would be expected, if we were to be studying mast cell degranulating compounds, independent of the drug itself.

Another problem of using acutely induced scratching as a model for itch is that the animals must be observed. This is a labour intensive procedure and highly inefficient. A limited number of mice can be observed at any once and if scratching is recorded and observed at a later date or observed at the time, a great deal of time is spent on a mundane task. Ideally, mouse scratching would be recorded automatically. Such a system has recently been developed whereby mice have a wire ring around the ankle and activity recorded due to the presence of a magnetic field, generated by copper wire and ferrite rods, in the cage in which the mouse sits. Movement of the metal rings, of a frequency related to that of scratching is identified (Elliott *et al.*, 2000). Of course the problem with such systems is that they are often likely to be costly and more invasive than simple observation. Further discussion and studies into this area can be found in chapter 4.

Drug induced scratching in animal models

The results gathered from experimentally induced scratching in animals is so far less complete than that which has been gained from human studies. This is primarily due to the fact that the research has not been carried out in the same depth, because of the problems I have discussed above. There have, however, been interesting findings that in some cases support, and in other cases contradict, what has been discovered in human studies.

Histamine has been shown to induce scratching in mice, as is known in humans. It is believed this is H₁ receptor, but also possibly H₂ receptor mediated (Inagaki *et al.*, 1999). The role of H₂ receptors in human itch is still very much inconclusive. As mentioned above, murine responses to histamine would appear to be strain related. More detailed discussion of histamine induced scratching in mice can be found in chapter 5 of this thesis. The mast cell degranulator, compound 48/80, also induces scratching in various strains of mice (Kuraishi *et al.*, 1995). As the human literature also suggests, this is H₁ receptor mediated and blocked using traditional antihistamines (Sugimoto *et al.*, 1998). Studies in guinea pigs also suggest histamine is pruritogenic when administered intradermally (Woodward *et al.*, 1995), however,

work in Sprague-dawley rats suggested scratching was not induced by histamine or compound 48/80 (Thomsen *et al.*, 2001).

As described previously substance P is pruritogenic in humans and this is assumed to operate through a histamine/mast cell mechanism. Work in mice has revealed a potentially separate mechanism. Substance P has been shown to evoke scratching in mice (Kuraishi *et al.*, 1995). Substance P has also been shown to induce scratching in mast cell deficient mice and can be blocked using an NK1 receptor antagonist; this is the substance P receptor that this evidence suggests may be located on the peripheral nerve transmitting the itch sensation (Andoh *et al.*, 1998). It has also been proposed that the NK1 receptor activity may not be peripheral but may be at the level of the spinal cord (Sakurada *et al.*, 1999), no such evidence exists to support this suggestion in humans. The possible role of the NK1 receptor is a novel one in the field of itch and although it may be a mouse specific mechanism it could be worth investigating in human studies.

Eicosanoids have also been studied in animal models of itch. Prostaglandin E₂ and leukotriene B₄ are pruritogenic in mice when administered intradermally (Andoh & Kuraishi, 2000). Prostaglandins E₂ and I₂ are pruritogenic when administered into the ocular surface of the guinea pig eye (Woodward *et al.*, 1996). Both these findings are contradictory to the evidence that exists in humans. Again the question is raised as to whether we should consider this to be species differences or a novel insight into a potential new mechanism of itch in humans, only with further research will we discover the answer.

Evidence has been published supporting the human literature for a pruritogenic evoking action of 5-HT in mice. Further to the human studies it was shown that 5-HT induced scratching was inhibited by 5-HT₂ but not 5-HT_{1A/3} receptor antagonists (Yamaguchi *et al.*, 1999).

Animal studies also support the human literature with regards to the central actions of opioids in inducing itch. Intracisternal administration of morphine or endogenous

endomorphins evokes scratching in mice (Kuraishi *et al.*, 2000; Tohda *et al.*, 1997; Yamaguchi *et al.*, 1998), similar to the effect of patients receiving an epidural injection. Intradermal injection of morphine does not elicit scratching (Kuraishi *et al.*, 2000).

Work using guinea pigs has suggested that PAF also induces itch in animals as well as humans. The work indicates that PAF may operate directly on sensory nerves and that the pruritic activity of PAF is not reduced by traditional histamine H₁ receptor antihistamines (Woodward *et al.*, 1995). A direct action of PAF on sensory nerves has not been described in the literature in humans.

Although the work in animal models is less comprehensive to date than that carried out in humans there is broad cross over in the drugs studied. The key to future work is to identify the differences between the two fields and establish simple species differences from potential drug targets for human studies.

Electrophysiological studies

In previous sections I have described studies in which recordings have been made from peripheral and spinal nerves. A small number of similar studies have been performed in rodents, but the problem with such studies is that evoking a neural signal does not tell us whether the animal would interpret that sensation as pain or itch? Nevertheless this technique can be useful in telling us whether a given drug activates peripheral nerves, and if it does, what other drugs activate the same afferent nerve fibres.

Peripheral C-fibres have been shown to increase firing directly to interleukin-2, a pruritogen in humans, in rats (Martin & Murphy, 1995). Histamine has been shown to activate spinal dorsal horn neurons in rats, although these nerves also appear to be sensitive to a range of other stimuli, including capsaicin (Carstens, 1997). The only work to date in mice indicates peripheral cutaneous nerves respond to 5-HT with a firing response of the same duration as the scratch response to 5-HT (Maekawa *et al.*, 2000). No evidence yet exists in rodents that there are specific nerves that respond to

pruritogenic, not algogenic drugs. If such nerves could be identified we could use electrophysiological studies in rodents to learn more about itch inducing, or itch-alleviating drugs, confident in the knowledge we were not inadvertently observing a pain associated response.

1.5 Aims of PhD Research Project

The aims of the studies in this thesis were designed to test the hypothesis that:

- Induction of scratching behaviour in mice is a useful and robust model for studying itch, which can be used to provide a guide to pruritic and antipruritic drugs relevant to man.

The techniques used in this thesis include both behavioural and in vivo electrophysiological procedures to examine drug-induced actions. Inducement of acute and chronic scratching in mice has been employed to study the actions of scratch inducing, and potential scratch alleviating, compounds.

The specific aims of this project are:

- To develop and validate a reliable and reproducible model of itch based on the induction of acute scratching in mice.
- Test putative mediators of scratching in this model.
- Use in vivo electrophysiological recordings from cutaneous nerves of mice to study neural responses to pruritogenic and algogenic drugs.
- Devise and develop an automated mechanism for the detection of scratching in mice.
- Examine the effect of novel itch-inducing drugs, established from murine studies, in human volunteers.

Chapter 2

Materials & Methods

The experiments described in this thesis were carried out in accordance with current Home Office regulations and guidelines as specified in the Scientific Procedures Act (1986). All experiments were performed under Project Licence number PPL: 60/2750 (Prof. D. McQueen) and, unless otherwise stated, my Personal Licence number PIL: 60/8073. The animals used for experiments described in this thesis were housed in licenced animal facilities within the School of Biomedical Clinical and Laboratory Sciences, in the Division of Neuroscience, University of Edinburgh, and experiments performed in licenced premises.

A total of 603 BalbC mice, 83 C57BL6 mice and 72 ICR mice were used. Animals were purchased from Charles River (UK) or Harlan (UK). Animals were housed under controlled light (07:00-19:00hrs) and temperature (22°C) with food and water available *ad libitum*. Animals were used for only one experiment, unless otherwise stated.

2.1 Acute Behavioural Studies

Scratch-Inducing Procedure

Scratching was induced in mice using an intra-dermal (i.d., 100µl) injection of pruritogen at the back of the neck, between the ears, using a 26G needle and standard 1ml syringe (BD Plastipak). During the injection procedure mice were restrained by scruffing by the neck.

The site at the back of the neck was chosen as it can only be reached by the hind limbs of the mouse and therefore scratching of the area can be easily distinguished from grooming behaviour, which utilises the forelimbs.

Measurement of Scratching

Mice were observed for a period of up to one hour post-injection, this period of time was reduced once the duration of a response was established. Animals were observed

and also filmed with a video camera (Vista, NCD 132) and their behaviour recorded using a VCR (Panasonic, NV-HD640).

The evoked scratching behaviour was measured and recorded. Scratching was defined as movements of the hind limbs to the injection site (back of the neck). Due to the speed of the scratching it was not possible by observation to count the number of individual scratch motions. For this reason, scratching was expressed as the number of 'bouts of scratching' evoked. A 'bout of scratching' was defined as three or more individual scratch movements of the hind paw to the area around the injection site. This separated the repetitive high frequency beating movement of a bout of scratching, from the 'pawing' of the area that was also sometimes observed.

At all times mice were also observed for any overt changes in behaviour that may have indicated discomfort or sickness caused by the injection.

2.2 Chronic Behavioural Studies

DNCB Model of Chronically Induced Scratching

In order to develop a model of scratching that is more akin to a clinical condition, chronic scratching was induced using the contact sensitising agent 2,4-Dinitrochlorobenzene (DNCB, Sigma, Poole, UK).

Mice in these experiments were shaved using clippers (Oster, small animal clipper, model A5-00) on day -1, on the back of the neck, and tail-marked. They were replaced into their cages, housed in groups of 3. DNCB was administered (1% in ethanol, topically) on days 0 and 3. DNCB was applied to the shaved area at the back of the neck, using a cotton bud that had been immersed in the DNCB solution. The cotton bud was rubbed over the skin for 5 seconds, and the mouse returned to the cage.

Mice were observed either once or twice daily until any skin reaction or scratching behaviour had subsided. Mice were observed for a period of ten minutes each time, and the level of scratching evoked was measured as described above. Written comments were recorded as to the condition of the skin (redness, swelling, bleeding etc.) on the back of the neck and the general behaviour of the mice.

Mice were treated with antipruritic drugs at the peak (days 6-11) of chronic scratching, either topically or intra-dermally. Further details of these protocols are described in chapter 7.2.

2.3 In Vivo Electrophysiological Recording from Cutaneous Afferent Nerves

Anaesthesia and Surgical Procedures

Mice were anaesthetised with urethane (1ml per 100g body weight, 20% w/v) intra-peritoneally, i.p. The depth of anaesthesia was tested using the toe-pinch withdrawal reflex, no experimentation occurred until this reflex was abolished.

Once anaesthetised, mice were shaved with clippers (Oster, small animal clipper, model A5-00), from the level between the ears to about one centimetre above the base of the tail, with the shaved area being about two centimetres wide.

Experiments were performed throughout on an automated heating blanket (Harvard Apparatus Limited, UK) to maintain body temperature at 37°C. Mice were placed on their front with their limbs secured to the operating table with tape. A midline incision was made in the shaved area, from between the ears, about 3 centimetres in length. The skin was dissected away from the underlying tissue, peeled back and held in place by stitching the skin to a small brass loop (see figure 2.1). This formed a pouch that was filled with heavy liquid paraffin oil (HLP) to ensure an electrically isolated recording system. A cutaneous nerve, running from the back of the mouse to the skin around the back of the neck, was then separated from the surrounding tissue

and split using fine forceps to produce as small a nerve filament as was possible. Typically this filament contained 1-3 afferent fibres. The nerve was crushed centrally using forceps to prevent efferent neural activity being detected by the distal recording electrodes.

Recording from a cutaneous nerve

Afferent neural activity was recorded by laying the dissected cutaneous nerve over bipolar platinum-iridium wire electrodes (see figure 2.2). The raw signal passed through a pre-amplifier (Neurolog, NL 103) and an amplifier (Neurolog, NL 105), producing a signal amplified by 10 000 times. The amplified signal was shown on an oscilloscope (Gould 1604) and digitised (Sony, Digital Audio Processor PCM-701ES). The digitised signal was filtered (Neurolog, NL 115) at 100Hz (low pass) and 1000Hz (high pass), before being passed through a voltage discriminator (Digitimer D.130) connected to a loud speaker. The filtered and voltage discriminated signal were passed through a Micro1401 interface (Cambridge Electronic Design, UK) and into a personal computer (Research Machines, Pentium III/500Hz) running Spike2 software (Cambridge Electronic Design, UK, v3.20). Action potentials were recorded on-line using both positive and negative trigger filters, keyboard markers were written into the file during recording in order to identify the exact time of injection. Files were saved as Spike2 data files for subsequent off-line analysis.

Identification of Receptor Field

Because a major objective of this thesis was the investigation of chemical mediators of pruritus, it was necessary to show that drugs were actually being injected into an area of skin innervated by the nerve from which recording was taking place. In order to prove this the skin around where the nerve passed into the skin was tested for mechanosensitivity using a hand held plastic probe. Firing of the units would show that the correct receptor field had correctly been identified; subsequent injections of drugs would be made at a site within this receptor field.

Drug Administration

Drugs were administered by an intra-dermal injection, into the area of skin held securely in place by the brass ring, into the area identified as in the receptor field of the nerve being recorded. Injections were in a volume of 100 μ l, administered using a 1ml syringe and a 26G needle.

Data Analysis

Data files were subsequently analysed using Spike2 software. The recorded waveform signal was scanned to identify action potential templates, representing individual nerve units. The signal was then rescanned and each unit shown on a separate channel. The signal was also displayed as a histogram, showing the total number of impulses of that unit in consecutive 1-second bins (see chapter 8 for examples of responses).

Data on individual units were expressed as the total number of impulses generated and as the frequency, in impulses per second. The 15-second period immediately preceding injection of the drug was analysed to provide a control. Responses to each injection are presented as the increase in the total number of impulses post injection relative to the pre-injection level, and likewise with the increase in frequency of impulses. The duration of onset of response and the total duration of response were also recorded.

2.4 Statistical Analysis

Data were analysed using Microsoft Excel 2000, GraphPad Prism (v3.02) and GraphPad InStat (v3.01) software.

Due to the nature of the work in this thesis (often relatively small sample sizes and large variability), non-parametric analysis parameters have been used. For comparison of two groups of means or medians a Mann-Whitney test was performed.

For paired results a Wilcoxon matched pairs test was used. For the comparison of three or more groups of means or medians, a Kruskal-Wallis (with Dunn's multiple comparison post-hoc analysis) was used. Where parametric analysis was deemed suitable, and distribution within groups appeared normal, a Student's paired (or unpaired) t-test was performed and for comparison of three or more groups a one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis was used.

In each case the null hypothesis (that difference between group means could arise due to natural variability) was rejected at the probability level of 0.05. Therefore, if the P value was less than 0.05 the null hypothesis was rejected and difference between groups were considered to be statistically significant.

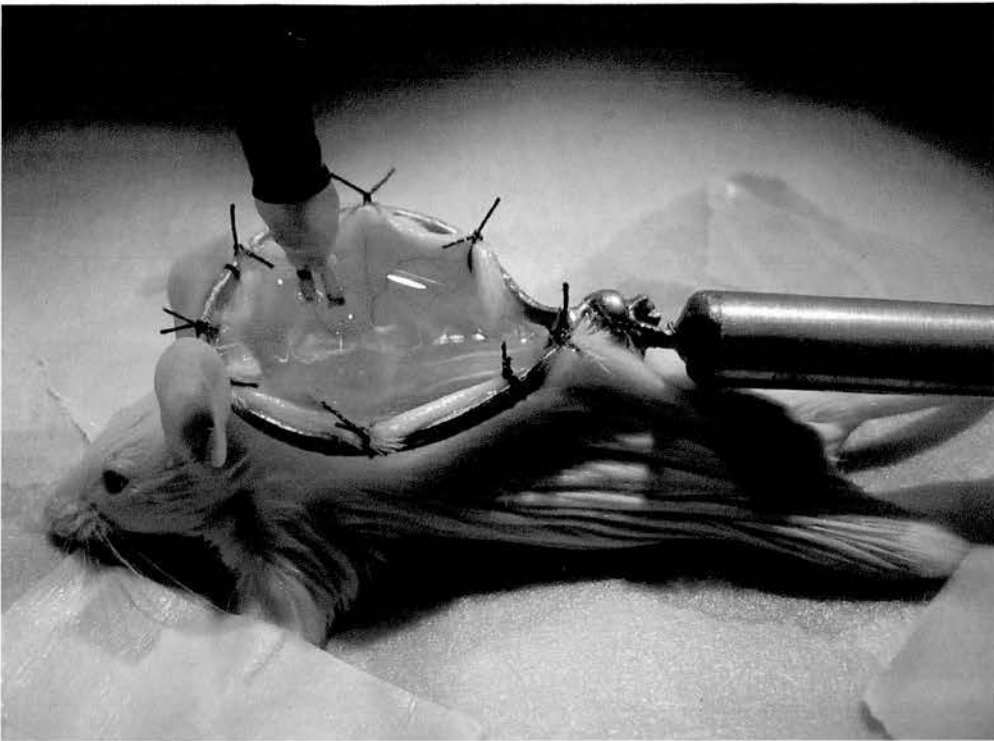


Figure 2.1 Image taken from a digital camera illustrating the surgical technique used for the electrophysiological studies. The diagram shows the skin of the back of the neck held by a brass ring to reveal the cutaneous nerves of the back.

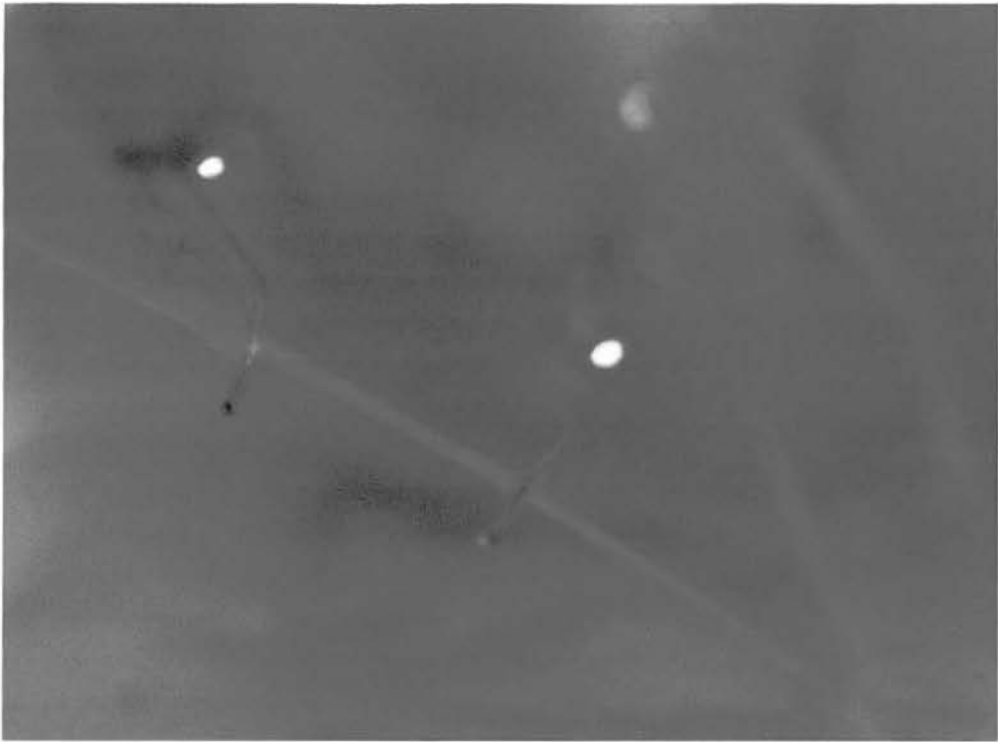


Figure 2.2 Image taken from a digital camera to illustrate the peripheral cutaneous nerve laying over the platinum-iridium electrodes for recording of action potentials.

Chapter 3

Development and Validation of a Model of Scratching in Mice

3.1 Introduction

The sensation of itch is a subjective one, and as such, even in humans, it is difficult to define clearly and concisely (Savin, 1998). We can never be certain that the same substance, in two different individuals, is evoking the same degree of sensation, or indeed the same sensation at all. For this reason, there are many methods used to try to quantify itch, with widely differing complexities. Methods used to measure itch in humans range from asking a subject to rate the intensity of the sensation using a visual analogue scale (Heyer *et al.*, 1998; Rees & Laidlaw, 1999), to recording activity of certain brain areas after an itchy stimulus (Hsieh *et al.*, 1994), to recording directly from what are thought to be 'itch nerves' (Schmelz *et al.*, 1997). These problems of measuring itch are substantial in humans, who have the ability to communicate the sensation and to understand what is being asked of them. Clearly, the problems of quantifying itch and being confident that the sensation being evoked is itch, are much greater in animal models, where oral communication is not possible. Having said this, animal models may be the most ethical and cost effective method of studying potential itch inducing or itch relieving drugs and their development is important.

Itch in human studies is commonly induced using one of two techniques, iontophoresis or intradermal injection of a pruritogenic substance. The more basic method of injecting pruritogen into the skin is widely used (Hagermark *et al.*, 1978) (Wahlgren *et al.*, 1995), however, there is an argument against using this method in itch studies. It is proposed that inducing a needle into the skin evokes a pain response that reduces the subsequent itch sensation (Keele & Armstrong, 1964). This suggestion is dependent on the gate-theory, which proposes that a painful stimulus acts centrally to reduce the activity of itch responsive nerves in that area of skin (Melzack & Wall, 1965). The second common method of inducing itch is iontophoresis (Heyer *et al.*, 1989; Weisshaar *et al.*, 1998), which involves passing a small electrical current between two electrodes placed on the skin, any ion-charged solution can be placed in a well in one of the electrodes and pass through the skin, into the dermis. Clearly this technique is more complex and it is difficult to ascertain

the exact dose of drug passing through to the skin, however, it is relatively pain free, non-invasive and ensures the drug is delivered as superficially as is possible. Both methods for inducing itch have been used in murine studies with no apparent adverse results detected with either technique (Kuraishi *et al.*, 1995; Laidlaw *et al.*, 2002).

Various elements of the experimental protocol used in animal studies into itch could potentially affect the results produced, each of these elements must be investigated if we are to be sure a model is robust and reproducible. It is essential to be confident that the procedures themselves are not capable of inducing scratching in mice, before we begin introducing drugs into the skin.

The basis of most models of itch involves the use of histamine, the first and still most commonly used pruritogen in research studies. The mechanism by which histamine evokes scratching in mice is further investigated in chapter 5 of this thesis. Histamine, since it was first described in the 1920's (Lewis, 1927) has been the most common experimental tool for inducing itch in human (Hagermark *et al.*, 1979; Keele & Armstrong, 1964) and in animal studies (Kuraishi *et al.*, 1995; Laidlaw *et al.*, 2002).

It has been proposed that, although histamine evokes scratching, there are other experimental variables to be considered. If the volume in which the drug is administered is too great there may be activation of pain sensing nerves, as described above, this could reduce the itch sensation due to the gate theory (Keele & Armstrong, 1964). Another possible variable of injecting pruritogens is the vehicle in which the drug is administered. Using distilled water could promote an ion imbalance, whereas using saline could create a change in pH (depending on the drug being investigated). It has been suggested that slight pH changes may evoke a sensation of itch in man (Keele & Armstrong, 1964).

If histamine is to be used as a standard pruritogen for studying itch, we must be aware of any unexpected responses following histamine administration. In humans, histamine has been shown to give a reduced itch response after repeated

administration; this is referred to as tachyphylaxis (Stahle-Backdahl *et al.*, 1988). Potentially, a lack of response to histamine may not simply be a consequence of that animal not being sensitive to histamine, but may be due to the protocol being used in the experiments.

Animal models need not necessarily always respond as in human models, however, aside from species differences we must also be aware of potential strain differences that may exist to various pruritogens (Inagaki *et al.*, 1999). Again, inconsistency in histamine responses may not be due to variability but may be due to the strain being used and may be an indication of distinct mechanisms operating. For example, release of histamine from mast cells has been shown to vary between different strains of mouse (Toda *et al.*, 1989). Strain differences in itch response need not be seen as a problem, but also an opportunity to learn more about separate mechanisms, which may relate favourably to those operating in man.

The response to a pruritogen in humans is scratching the afflicted area. Without the ability to communicate with animals we can't be sure that they feel the same sensation or respond to that sensation in the same way. It has been reported that arthritic rats scratch, yet this sensation would be expected to be pain, not itch (De Castro-Costa *et al.*, 1987). Animals may not only scratch when they feel itchy, this has caused some authors to question whether the study of itch based on scratching induced in animals is a sound basis for quantifying itch (McMahon & Koltzenburg, 1992). Evidence exists to support the argument that animals scratch only in response to itch. Mice have been shown to scratch to the pruritogens (in humans) compound 48/80 and substance P, but not to the algogenic compounds capsaicin and formalin (Kuraishi *et al.*, 1995). It is important to be aware of any changes in animal behaviour when developing such models as we can't assume what behavioural response an individual animal will give to any particular drug.

The purpose of the studies in this section of the thesis is to develop and validate a murine model of itch. The experiments in this chapter were performed in order to determine the ability to evoke scratching in mice, and whether or not we can suggest

that this behavioural response correlates with itch in man. Whether or not the experimental procedures used to generate this scratching behaviour provide a robust and reproducible model for similar studies into murine scratching was also investigated.

3.2 Materials and Methods

The itch-inducing procedure and measurement of scratching in this chapter are as previously described in chapter 2.1, unless otherwise stated. Experiments in this chapter were conducted in a total of 156 mice: 125 female BalbC mice, 8 male BalbC mice, 17 female ICR mice and 6 female C57/Bl6 mice.

3.3 Results

Scratching evoked by the itch-inducing procedure

The scratch inducing abilities of various experimental procedures were investigated in a total of 30 female BalbC mice (see figure 3.1). Scratching was measured, over a 20-minute period, following; no experimental procedure; scruffing of the mice; insertion of needle into the skin; injection of distilled water into the skin (0.1ml, i.d.); injection of saline (0.1ml, i.d.) and injection of phosphate buffered saline (PBS, 0.1ml, i.d.). A low level of scratching was observed in each experimental group, but there was no statistically significant difference between the means of any of the individual groups (Kruskal-Wallis test with Dunn's multiple comparison post-test, $P > 0.05$ between all groups).

Histamine evoked scratching

Histamine-evoked scratching was dose-dependent in all eight BalbC mice studied, as summarised in figure 3.2. Scratching was not obtained at lower doses (up to 0.8 μ mol), but was consistently observed with higher doses, reaching a maximum at 26 μ mol histamine. Above this dose there was no further increase, and often a

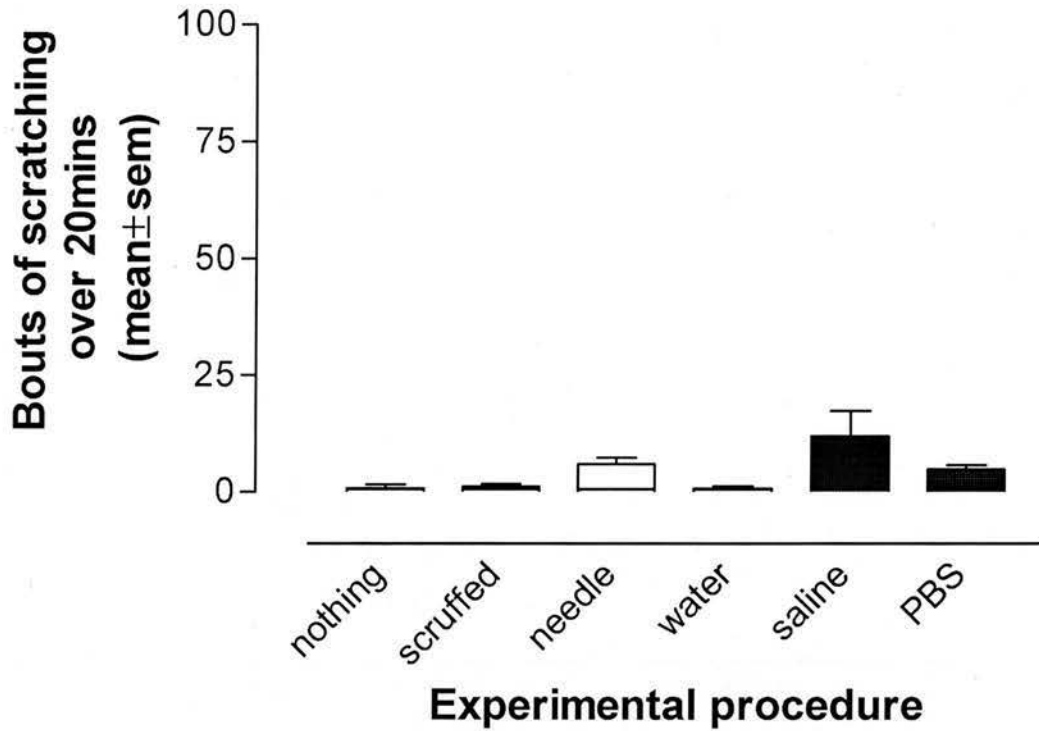


Figure 3.1 Scratching evoked by the experimental protocol used in the development of a murine model of scratching in female BalbC mice. There was no significant variation in mean response between unhandled mice ('nothing', $n=5$), scruffed mice ($n=10$), mice inserted with a needle ($n=10$), mice receiving water (0.1ml, i.d. $n=5$), mice receiving saline (0.1ml, i.d. $n=8$) and mice receiving PBS (0.1ml, i.d. $n=12$, $P>0.05$, Kruskal-Wallis test with Dunn's multiple comparison post-test).

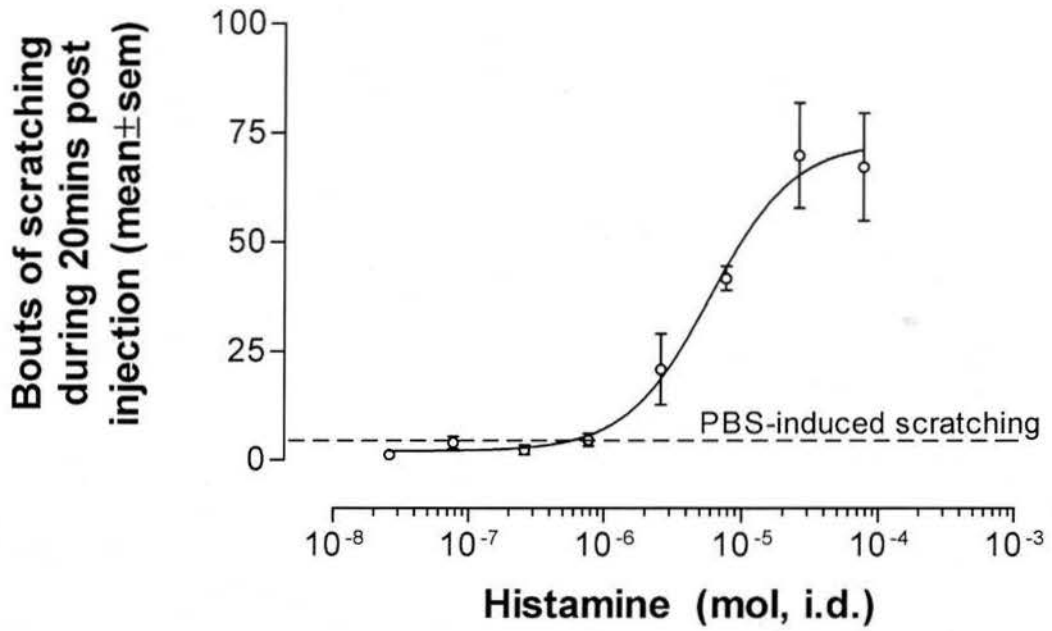


Figure 3.2 Pooled data for histamine-induced scratching in female BalbC mice during the 20-minute post injection period ($n=4-8$). Histamine caused dose-dependent scratching. Dashed line represents the mean level of scratching induced by PBS, which served as a control (5 ± 1 bouts of scratching, $n=12$).

decrease, in scratching. Histamine-induced scratching reached a maximum response of 73 ± 12 bouts (mean \pm sem, median=70), with a mean apparent ED_{50} of $5.8 \mu\text{mol}$.

Effect of various vehicle solvents on histamine evoked scratching

Histamine-induced scratching ($8 \mu\text{mol}$, i.d.) was shown to be reproducible and independent of the vehicle used (see figure 3.3). Mean scratching was not significantly different whether PBS (pH 6.8) or distilled water (pH 5.6) vehicle was used (Mann Whitney test, $P > 0.05$). Saline vehicle, which had a pH between the two values for PBS and water (pH 6.1), also showed no significant difference in scratching.

Effect of injection volume on histamine evoked scratching

The effects of a sub-threshold dose of histamine ($0.8 \mu\text{mol}$, i.d.), when injected in a volume of $100 \mu\text{l}$, were compared to the same dose of histamine, administered in a volume of $10 \mu\text{l}$. Significant levels of scratching were not observed in either group ($100 \mu\text{l}$; 0 ± 0 bouts of scratching, (median=0), $10 \mu\text{l}$; 1 ± 1 bouts of scratching (median=0), mean \pm sem, $n=3$ in each group). The same pattern was observed with a dose of $3 \mu\text{mol}$ histamine (a dose at the base of the dose response curve); $100 \mu\text{l}$ -scratching response was 4 ± 4 bouts (median=0), $10 \mu\text{l}$ -scratching response was 1 ± 0 bouts (median=1, $n=3$ in each group).

Effect of mouse gender on histamine evoked scratching

Scratching was induced using histamine in eight male BalbC mice and compared to the responses of female mice, described above. Histamine caused dose-dependent scratching in male mice as previously observed in female mice (see figure 3.4). In male mice, histamine-induced scratching reached a maximum of 65 ± 5 bouts (mean \pm sem, median=63), with a mean apparent ED_{50} of $5.7 \mu\text{mol}$. These values are very similar to those measured from the female mice (73 ± 12 bouts; mean apparent ED_{50} of $5.8 \mu\text{mol}$), indicating no gender differences in histamine induced scratching response in BalbC mice.

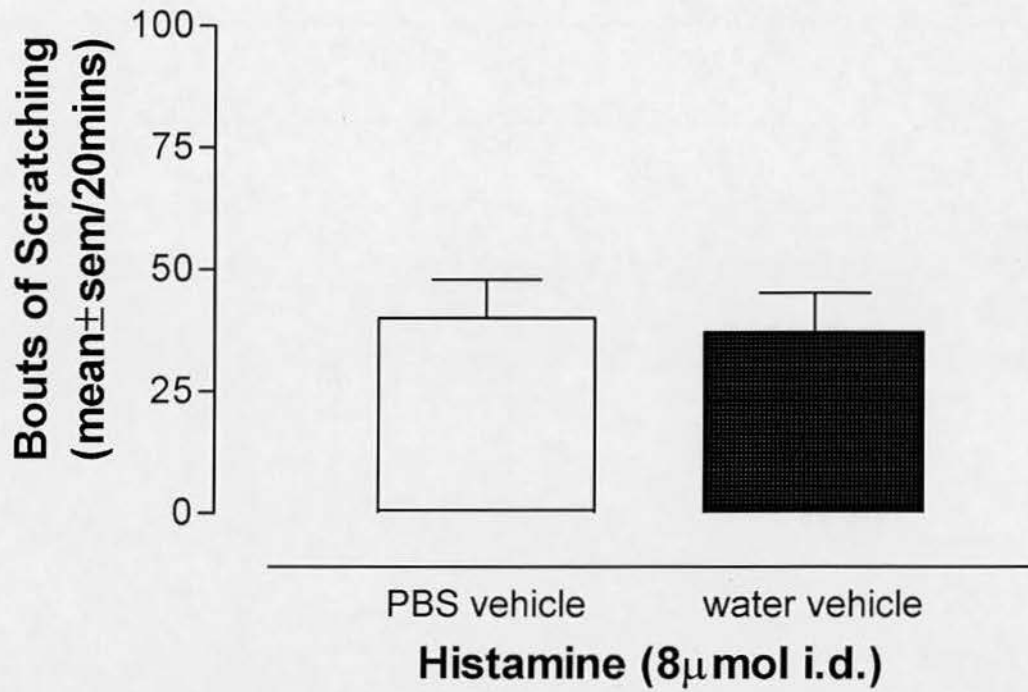


Figure 3.3 PBS versus distilled water vehicle does not affect histamine (8μmol, i.d) evoked scratching in female BalbC mice ($P>0.05$, Mann-Whitney test, $n=7$ in each group).

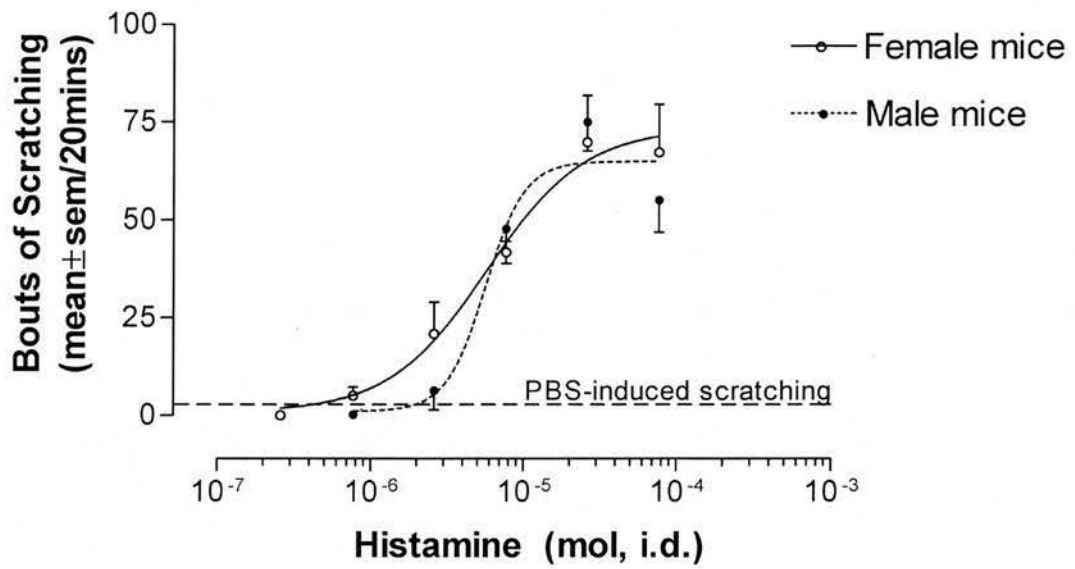


Figure 3.4 Pooled data for histamine-induced scratching in male BalbC mice (n=8) compared with female BalbC mice (data as in figure 3.2). Histamine caused dose-dependent scratching in both male and female mice. Dashed line represents the mean level of scratching induced by PBS, which served as a control (5±1 bouts of scratching, n=12).

Characteristics of histamine evoked scratching

Time course of scratching response

The level of scratching evoked by three doses of histamine (8-80 μ mol, i.d) was measured over the course of 40 minutes. Scratching primarily occurs in the first 10 minutes following injection of histamine, regardless of dose administered (see figure 3.5a). Histamine (8 μ mol) induced a total of 42 ± 8 bouts of scratching, 38 ± 4 of which occurred during the first 10 minute period ($n=8$). Histamine (30 μ mol) evoked a total of 70 ± 12 bouts of scratching, 50 ± 7 bouts of which occurred in the first 10 minute period. Finally, histamine (80 μ mol) induced a total of 67 ± 12 bouts of scratching, 44 ± 10 bouts of which occurred in the first 10 minutes. Very little scratching occurs after 20 minutes after any of the histamine doses, although to the higher two doses there was a prolonged scratching response from the first 10 minutes into the second 10-minute period. More detailed studies indicated that scratching occurred throughout the first 10-minute period following histamine injection (8 μ mol, $n=6$, see figure 3.5b). Most scratching occurred in the period 4-6 minutes post injection.

Sensitising and desensitising effects of histamine

The effects of moderate and high doses of histamine on subsequent histamine responses were investigated in order to evaluate whether these doses were causing sensitisation or desensitisation. A high dose of histamine (30 μ mol, i.d) elicited a scratching response of 70 ± 12 bouts of scratching, median=66, in the earlier dose response studies, when this dose was administered at the end of the experimental procedure. If a similar dose of histamine was administered before any other procedure the scratching response was significantly reduced, 37 ± 5 bouts of scratching ($n=6$ in each group, $P<0.05$, unpaired t-test, median=43.5), indicating that the earlier lower histamine doses imposed a slight sensitising effect on later injections. Inversely, a high dose of histamine (30 μ mol, i.d) administered prior to a lower dose of histamine (8 μ mol, i.d) reduced scratching responses, indicating that the higher doses of histamine imposed a desensitising effect on later injections. Control scratching responses to histamine (8 μ mol), prior to any other experimental procedure, were 49 ± 5 bouts ($n=6$ in each group, median=50). Scratching responses

to histamine (8 μ mol) in mice, one hour after a high dose of histamine (30 μ mol) was significantly lower, 12 \pm 4 bouts (n=6, $P<0.05$, unpaired t-test, median=10).

Further studies into the desensitising effects of histamine on subsequent doses of histamine were carried out in 12 ICR mice. Female ICR mice were used in repeat experiments at weekly intervals and their responses to histamine studied. ICR mice showed reduced histamine responses, to a range of doses, over successive weeks. Histamine (30nmol, i.d) evoked 23 \pm 4 bouts of scratching in week one, this fell to 22 \pm 11 bouts in week two and 2 \pm 1 bouts in week three (n=4). Similar patterns of diminishing scratching response histamine over successive weeks were identified in a range of doses from 8-800nmol.

Strain dependent differences to histamine evoked scratching

Scratching evoked by histamine was studied in female BalbC (as previously described above), ICR and C57/Bl6 mice. In each strain histamine was shown to induce dose dependent scratching. As discussed earlier BalbC mice respond to histamine with a mean apparent ED₅₀ of 5.8 μ mol (n=8). ICR mice respond to a significantly lower dose of histamine than BalbC mice, with a mean apparent ED₅₀ of 0.2 μ mol ($P<0.05$, Kruskal-Wallis test with Dunn's multiple comparison post test, n=5, see figure 3.6). Interestingly, this dose (0.2 μ mol) was shown to be unable to evoke scratching in any female BalbC mice studied (as can be seen in figure 3.2). Female C57/Bl6 mice also responded dose dependently to histamine with a mean apparent ED₅₀ of 1.3 μ mol (n=6, see figure 3.6). The mean apparent ED₅₀ of C57/Bl6 mice was not significantly different from either the BalbC or ICR strains, however, the trend suggests they respond to histamine in an intermediate dose range to that identified in the other two strains investigated ($P>0.05$, Kruskal-Wallis test with Dunn's multiple comparison post test).

The maximum scratching evoked by histamine was significantly greater in C57/Bl6 mice than in the other two strains investigated. Scratching reached a maximum level of 131 \pm 8 (mean \pm sem) bouts, over 20 minutes post-injection, in C57/Bl6 mice (n=6). This was significantly higher than that seen in BalbC mice (73 \pm 12, n=8) or ICR mice

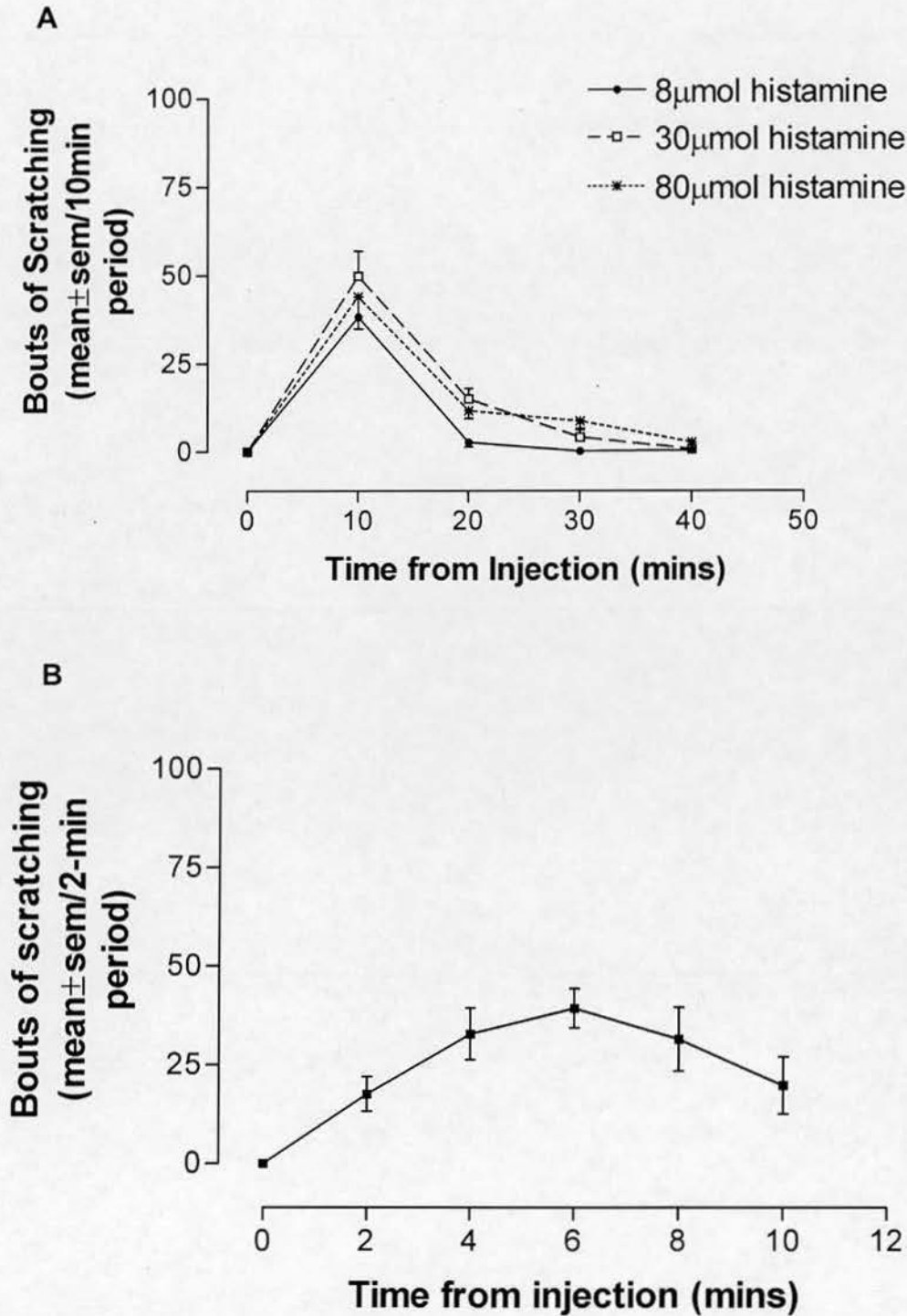


Figure 3.5 Time course of histamine (i.d.) evoked scratching in female BalbC mice over A, a 40-minute period and B, a ten-minute period. A; scratching occurs primarily in the first ten-minute period at doses of 8 μ mol (n=8), 30 μ mol (n=6) and 80 μ mol (n=4). B; scratching peaks in the time period 4-6 minutes post injection (8 μ mol, n=6).

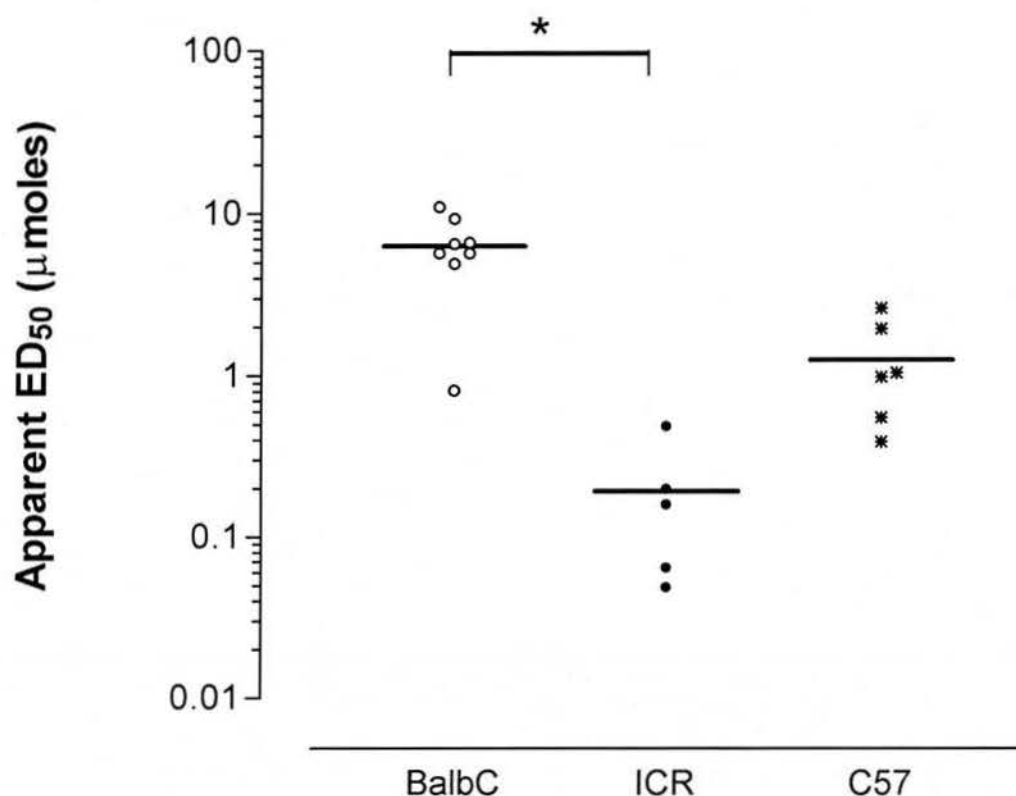


Figure 3.6 Apparent ED₅₀s for histamine in female BalbC (n=8), ICR (n=5) and C57/Bl6 mice (n=6). Mean apparent ED₅₀ was significantly lower in BalbC than in ICR mice (*P<0.05, Kruskal-Wallis test with Dunn's multiple comparison post test). Mean apparent ED₅₀ in C57/Bl6 mice appeared to be intermediate between the other two strains, but this was not statistically significant (P>0.05, Kruskal-Wallis test with Dunn's multiple comparison post test). Bars show mean value.

(66 ± 16 , $n=5$, $P<0.05$, Kruskal-Wallis test with Dunn's multiple comparison post test).

Scratch inducing properties of 'algogenic' substances

A variety of drugs assumed to evoke a sensation of pain, was investigated for their scratch-inducing capabilities. Hydrochloric acid (HCl, 32%), formalin (2-10%), bradykinin (0.3-33nmol) and capsaicin (1-33nmol) were studied. The scratch evoking properties of these drugs are shown in figure 3.7a, which shows the doses of bradykinin and capsaicin that evoked the greatest level of scratching. HCl and formalin (10%) did not cause scratching in female BalbC mice. Formalin (2%) and bradykinin (9nmol) evoked a small scratching response, which did not differ significantly from that evoked by PBS. Capsaicin (33nmol) evoked a scratching response significantly greater than that evoked by PBS ($P<0.05$, Mann-Whitney test).

Time course of capsaicin-induced scratching

Histamine evokes scratching that occurs mainly in the first 10 minutes post injection, as described above. The pattern of the scratching response to capsaicin was also investigated (see figure 3.7b): very little of the scratching occurred during the first 10 minutes. Total scratching evoked was 57 ± 14 (mean \pm sem, $n=4$), of which only 3 ± 3 occurred within the first 10 minutes post injection. Unlike histamine induced scratching, capsaicin induced scratching persisted for 40 minutes.

Behavioural responses

Intradermal administration of HCl and formalin (10%) evoked a behavioural response that was quite distinct from that after histamine. The only obvious behavioural change following histamine injection was the onset of scratching. However, injection of HCl and formalin was associated with vocalisation in all mice. Following injection of these agents the mice exhibited prolonged 'crouching' and 'cowering'. This behavioural response persisted in each case until the animals were killed. Following injection of HCl and formalin (10%) mice were killed after 10 minutes to prevent undue discomfort and pain that was adjudged to be occurring.

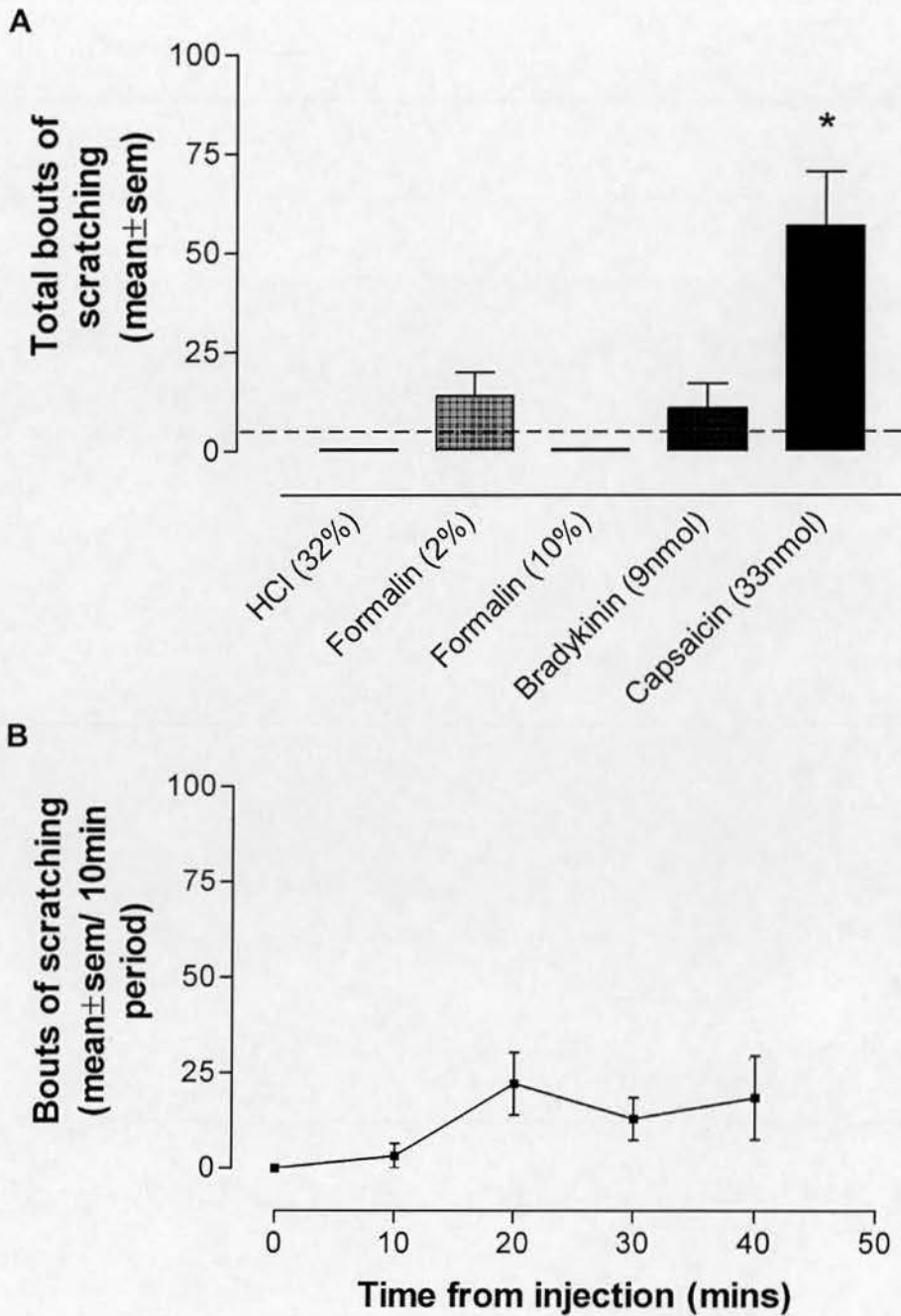


Figure 3.7 A; Scratching evoked by the algogens HCl ($n=4$), formalin (2%, $n=3$), formalin (10%, $n=4$) and bradykinin ($n=4$) was not significantly greater than that evoked by PBS in female BalbC mice ($P>0.05$, Kruskal-Wallis test with Dunn's multiple comparison post test). Scratching evoked by capsaicin ($n=4$) was significantly greater than that evoked by PBS (* $P<0.05$, Mann-Whitney test). (Dashed line illustrates scratching induced by PBS). B; time course of capsaicin induced scratching over a forty-minute period. Scratching began after the first 10-minutes after injection and lasted for 40 minutes.

3.4 Discussion

The results from this section of the study show that scratching can be evoked in mice in a reliable and reproducible manner. The data also indicate that quantifying the scratching behaviour evoked can be used as a method of studying itch objectively in these animals.

The initial experiments of this study were focussed on establishing whether the devised protocol for inducing scratching was suitable as a method for studying itch. Scratching in mice was not evoked by any of the experimental control procedures used. Mice were shown not to scratch prior to handling, while neither scruffing the animal or insertion of a needle evoked scratching response. Water, saline and PBS induced a small scratching response, but these responses were negligible when compared to responses generated by the pruritogen, histamine, as described below. These simple control experiments ensure that the induction of scratching was a response to agents being administered to the skin and not due to the model itself.

The method used for inducing scratching in these studies involved intradermal injections of pruritogen. The use of iontophoresis in itch research is common in both human and animal models (Hagermark *et al.*, 1978; Laidlaw *et al.*, 2002; Wahlgren *et al.*, 1995). Iontophoresis has many advantages, mainly that it does not usually evoke a pain response and that the drug is delivered superficially. Despite the benefits of iontophoresis it was not used in these studies due to two main reasons. Iontophoresis in humans is made easy due to the lack of movement of the subject. The use of animals, which are in many cases only slightly bigger than the equipment itself, provides problems of restraining the animal to ensure the drug can pass through to the skin without the electrodes moving away from the desired point. The second reason iontophoresis not being used was that estimating the dose of drug being administered is less precise. The studies in this thesis include a dose response element, which is easier to estimate using the intradermal injection method.

Mainly due to its historical role and wider reporting in the literature, the basis of the scratching model developed was using histamine. Histamine is recognised as an inducer of itch in humans (Hagermark *et al.*, 1979; Keele & Armstrong, 1964; Lewis, 1927). The strain of mouse selected for the development of the model was made for various reasons. The BalbC mouse was recognised as a standard laboratory strain that had been used in research into itch that had been previously presented in the literature (Inagaki *et al.*, 2001; Inagaki *et al.*, 2000; Laidlaw *et al.*, 2002; Sugimoto *et al.*, 1998). This strain was relatively cheap and readily available, with the known ability to scratch following drug application. Results from this study show histamine evokes a dose dependent scratching response in mice, as previously described in the literature (Inagaki *et al.*, 2001; Laidlaw *et al.*, 2002), but contrary to some evidence (Kuraishi *et al.*, 1995). The results of this study also show that the scratching response is independent of mouse sex. Female mice were used in these studies, primarily for ease of housing, but it would appear a model could also be based on male mice.

The evidence from these studies indicate that for a given dose of histamine, the scratching is independent of the vehicle used; water, PBS or saline. This evidence shows that changes in ion balance or pH changes do not significantly affect the scratching response, as was previously suggested (Keele & Armstrong, 1964). Despite this finding, PBS was used as a standard vehicle for all intradermal injections where possible.

Changes in the volume of vehicle in which the drug was injected might also affect scratching response (Keele & Armstrong, 1964). This evidence proposes that the itch sensation may be reduced if the volume of injection is too great, due to the activation of pain nerves, reducing the activity of itch nerves via the 'gate-theory' (Melzack & Wall, 1965). In order to test this suggestion a dose found to be sub-threshold, and another dose at the base of the histamine dose response curve were administered in a lower volume (10 versus 100 μ l). The results indicate that there is no increase in scratching response if the histamine is administered in a smaller volume.

The temporal characteristics of scratching in response to histamine were examined in greater detail. Histamine evoked scratching lasted a maximum of forty minutes. However, the majority of scratching occurred within the first ten-minute period. The peak of scratching occurred 4-6 minutes after the injection of histamine. The pattern of scratching in the first ten-minute period was true of all doses, although with higher doses, where more total scratching occurred, there was still scratching in the second ten-minute period. These findings support the literature in both human and animal models that histamine induced itch lasts for 10 to 20 minutes (Hagermark *et al.*, 1979; Inagaki *et al.*, 1999; Keele & Armstrong, 1964; Laidlaw *et al.*, 2002).

The results of this study show high doses of histamine cause a desensitisation to future histamine injections. These results support the evidence that in humans, histamine causes tachyphylaxis after repeat administration (Stahle-Backdahl *et al.*, 1988). Doses of histamine at the lower end of the dose response curve did not cause desensitisation, and in fact seem to sensitise the mice to subsequent histamine application. Studies in which mice were used for multiple experiments at weekly intervals showed that histamine response was reduced after administration over successive weeks. For this reason, all future experiments used mice for one experiment only. Repeat use of animals would have proved beneficial for not only economic reasons but also would have enabled each animal to act as its own control over a period of time. Unfortunately, the evidence from these experiments indicated that this would not be possible.

The effect the particular strain of mouse has on histamine evoked scratching response was investigated in two other common laboratory strains; ICR and C57/Bl6 mice. Both strains had previously been described in the literature in itch studies (Andoh *et al.*, 1998; Kuraishi *et al.*, 1995). The results from this study supports previous evidence that there are significant strain dependent differences in histamine evoked scratching responses. The dose of histamine that induced half the maximum scratching response (mean apparent ED₅₀) was significantly lower in the ICR strain in comparison with the BalbC strain (approximately 30 times). The C57/Bl6 strain appeared to have histamine sensitivity between the other two strains studied. The

reason for these differing sensitivities is not clear, but could be due to separate histamine receptor subtypes being present, or different behavioural responses to the same sensation. Further studies continued using the BalbC strain. Although more sensitive, the ICR and C57/Bl6 strains provided more variable responses, not ideally suited to behavioural studies in which variability was already a significant obstacle. It is also important to note that histamine dose sensitivity in BalbC mice from this study is approximately 100 times lower than in humans (Hagermark *et al.*, 1979). This illustrates that species differences should not be forgotten when interpreting the importance of animal models. The reason for the difference between species is unknown. The mechanism of histamine mediated scratching is further described in chapter 5 of this thesis.

It has been suggested that scratching in mice may not provide a sound basis for estimating itch (McMahon & Koltzenburg, 1992). This is partly based on the observation that arthritic rats appear to scratch more than non-arthritic rats, and that this would be expected to be a pain response, not itch (De Castro-Costa *et al.*, 1987). The results from this study indicate that compounds thought to evoke pain (HCl, formalin and bradykinin) do not evoke scratching at a level greater than that induced by PBS. This evidence supports earlier findings in mice that pruritogenic, not algogenic drugs evoke scratching in mice (Kuraishi *et al.*, 1995). An exception to this evidence would appear to be that capsaicin evoked scratching in mice in this study. One explanation would be that mice scratch to some but not all pain stimuli, although this would seem unlikely. Another explanation is that capsaicin is capable of evoking the sensation of itch, possibly as well as that of pain, a suggestion that previously been made in the literature (Lynn, 1992). One major argument against this explanation would be the gate theory described above, that a pain response has the ability to reduce an itch response centrally, therefore eliminating the need to scratch. The results of this study provide a clue that this may not necessarily be the case. Unlike histamine-evoked scratching, capsaicin induced scratching does not occur in the first ten minutes post injection; in fact the scratching begins after this time. This provides the possibility that pain does reduce the itch response in the early stages, but as the pain response declines, the itch response becomes prevalent and scratching

occurs. A second possibility would be that capsaicin evokes only pain, and no itch, but that 'pain-induced scratching' occurs over a time course distinct from that of itch-induced scratching. Either of these explanations does not conceal the fact that the model developed in this study can differentiate between a pain response and an itch response.

In summary, the results from this study indicate that the murine model developed can be effectively used to induce and quantify the level of itch. The model, based on the use of intradermally injected histamine and recording of the scratching evoked, provides a robust and reproducible model, on which a range of other putative mediators of itch could be investigated.

Chapter 4

Development of an Automated Scratch Detector

4.1 Introduction

The subjective nature of the itch leads to inevitable problems in quantification of the sensation. In humans, these problems are overcome primarily by the patient expressing the degree of itch using an arbitrary scale, such as a visual analogue scale (Greaves & Wall, 1996; Heyer *et al.*, 1989; Rees & Laidlaw, 1999; Wahlgren *et al.*, 1989). In animal models the quantification of itch cannot be reported in such a way. As described in Chapter 3, animal models rely on the recording of scratching behaviour as a measure of itch.

Manual measurement of scratching in mice involves video recording of animals and watching of the video at a later date, or observation of the animals at the time of investigation. Either of these approaches involves an individual investigator watching the animals in 'real time', counting the number of scratches. This approach is extremely time consuming and an unproductive use of research time. Other drawbacks of such an approach are that few animals can be observed. Pilot studies established that it was simply not possible to measure the scratching of any more than four individual animals at any one time. Clearly this is a restriction on the productivity of the research. Another disadvantage of manual observation of scratching is that in real-time only 'bouts of scratching' can be identified (see chapter 2.1). Without the use of slow motion videoing it is not possible visually to discern the individual number of scratch movements made by the animal. However, to watch each individual animal in slow motion over the course of the study would simply add to the labour intensity and lack of productivity.

In order to reduce the labour intensity and increase efficiency it would be preferable to automate the detection of scratching in individual animals. Similar developments have been attempted in human studies. Detectors of limb movement have been placed on the arms and legs of pruritic patients that record the total movement of each limb over the course of a night (Summerfield & Welch, 1980). This system, although not a direct measure of scratching, provides an objective valuation of itch when compared to the level of movement in non-pruritic subjects. Two automated

detectors of scratching in mice have previously been described in the literature. The first system attached aluminium wire rings to the hind legs of mice and placed the animals in cages that were surrounded by ferrite rods and copper coils. Movement of the hind limb, and therefore the wire ring, evoked an electrical current in the rod and coil. This electrical current was computer analysed and electrical pattern similar in intensity to that of scratching could be identified (Elliott *et al.*, 2000). A second system involved the insertion of small magnets into the hind paws of mice and the animals were placed in a cage surrounded by a coil. Again, the movement of the hind paw, and magnet, evoked an electrical current that was identified by computer to search for patterns related to that of scratching, based on the intensity and frequency of the movement (Inagaki *et al.*, 2003).

The purpose the present study was to develop and validate a relatively simple, inexpensive and non-invasive automated detector of scratching in mice based on the repetitive movements that occur as a mouse scratches the back of the neck using a hind paw.

4.2 Materials and Methods

Force Platform

An inexpensive force platform was constructed using two parallel aluminium plates (20cm x 30cm) joined by plastic hinges at one end and separated at the other end by a force transducer (see Figure 4.1C). This simple design of force platform exhibits varying sensitivity to movement forces depending on distance of the animal from the hinge but in practice that has little effect, as the activities being monitored are restricted to a narrow area of the upper plate distal from the hinge. Other factors, such as the direction of the repetitive movement being monitored, also affect the forces measured by the platform. More complex designs of force platform were considered during the early stages of development. For example, using three force transducers it is possible to obtain an accurate measure of the vertical force and also

the position of the animal in the cage. In practice, the much simpler design described here provides appropriate data for use in the present studies.

The transducer output is amplified by a bridge amplifier (see Figure 4.2). The offset and gain of this stage may be altered by front panel controls on the repetitive detector unit. The output of the bridge amplifier is filtered by a 20Hz 8th order switched capacitor low pass filter. The buffered analogue output from these stages is available externally.

Low weight compressed polystyrene cages were placed on top of the platform, held in place by small steel pins. The mouse cages were designed to be as low weight as possible in order for the movement signal generated by the mouse to be as large as possible, relative to the background signal evoked.

For routine testing, a simple test 'mouse' phantom was constructed using a small electric motor with an eccentric weight of a few grams mounted on its shaft. The phantom was self-contained with its own battery and its speed could be adjusted to confirm the correct triggering of the repetitive event detector.

Theory

In designing the system we considered the forces that would be generated by a repetitive movement of a small mass such as the hind limb of a mouse. Consider a mass (m) of 1g moving vertically sinusoidally with a frequency (f) of 12Hz and amplitude (D) of 5mm.

Displacement $x = D \sin(2\pi f t)$ where t is the elapsed time (sec)

$$\text{Acceleration } a = -4D\pi^2 f^2 \sin(2\pi f t)$$

The corresponding force (F) measured by the force platform would be

$$F = m a$$

The amplitude of this force would be

$$|F| = 4 m D \pi^2 f^2 = 4 \times 10^{-3} \times 5 \times 10^{-3} \times 144 \times \pi^2 = 28.4 \text{ mN} = 2.9 \text{ gf}$$

This would be observed as 5.8gf peak to peak.

If the sinusoidal movement was not vertical but at an angle of θ to the vertical then the force exerted on the platform would be reduced by the factor $\cos(\theta)$.

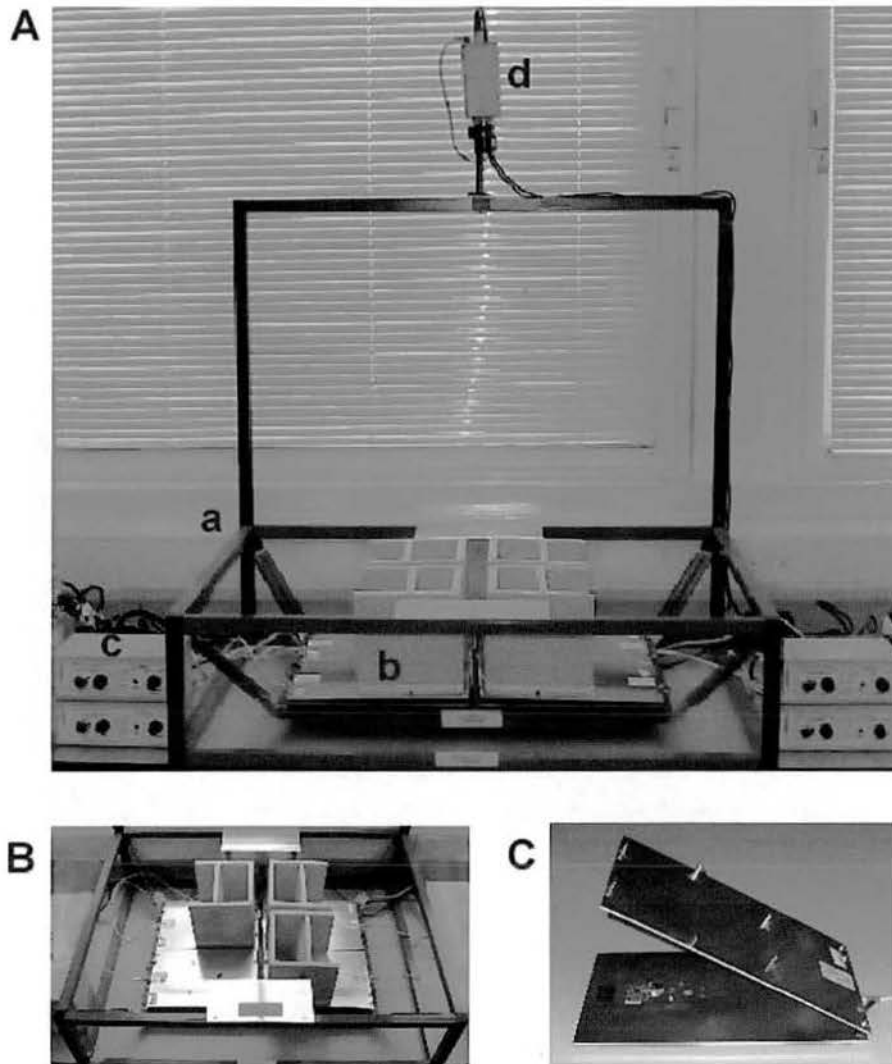


Figure 4.1 A: Overall view of apparatus showing the supporting framework (a) for the anti-vibration table (b), the four force platforms with animal boxes (b), the associated repetitive movement detector electronics (c) and the overhead video camera (d). B: Aerial view of the four force platforms with one animal box removed. Studies could be conducted on four mice simultaneously. C: Force platform with hinged plates separated to show the force transducer.

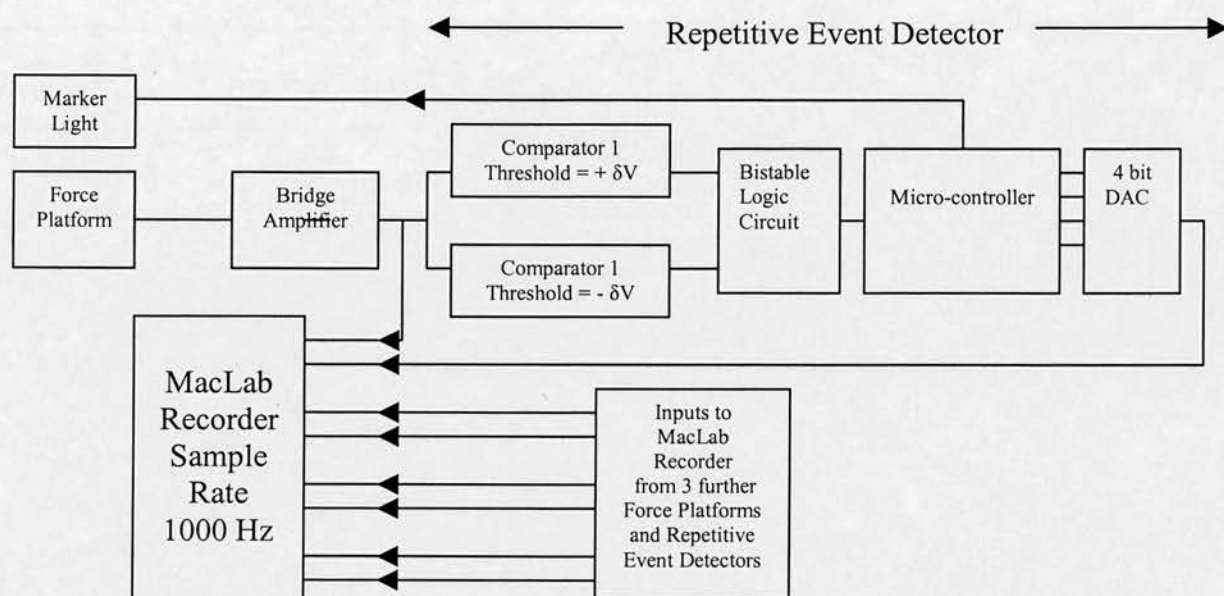


Figure 4.2 Flow diagram illustrating the data collection system used in the automated scratch detector.

Repetitive Event Detector

The filtered output signal from the force platform is also applied to two comparators (figure 4.2). One comparator was set at δV volts above zero and the other at δV volts below zero. δV may be adjusted, by a front panel control, over the range of 0mV to 400mV. The comparator outputs are applied to a bistable logic circuit such that its output switches to a logic '1' when the signal exceeds the upper threshold and switches to a logic '0' when the signal goes below the lower threshold. The bistable output is applied to a data input of a PIC16F84 micro-controller.

The micro-controller was programmed to identify periodic intervals in the bistable output signal that corresponded to repetitive frequencies in the range 8Hz to 20Hz. The program does not acknowledge a single acceptable interval but requires a minimum of two such intervals, after which it accumulates the intervals until two incorrect intervals are detected (the total number of acceptable intervals received relates directly to the number of scratch movements identified by the micro-controller). The lowest acceptable frequency may be selected from either 8Hz, 9Hz, 10Hz or 11Hz by a front panel control. This variation in the frequency filters allows us to identify movement of varying frequency of scratch movement, which may be seen between individual mice. To assist in the analysis, the repetitive count is fed through a 4 bit digital to analogue converter (DAC) the output of which was recorded with the filtered analogue data on a PowerLab system. The number of intervals detected is readily determined later by examining the staircase waveform from the DAC. An external white light, situated beside the cage, was provided for marking any detected events onto video recordings that were made of the studies, to assist in the locating of the scratching behaviour when observing the videotape at a later date.

Anti-vibration Table

The force platform proved to be extremely sensitive and it detected vibrations from the steel frame building in which it was used. In addition, vibration caused by staff moving around the laboratory, lifts and air conditioning systems also contributed considerable levels of unwanted 'noise' to the platform signal, confounding measurements. After some tests, an anti-vibration table was constructed. This consisted of a 20kg rectangular steel plate supported within a tubular steel frame by

steel springs at 45° to the horizontal, attached to each corner of the plate. The anti-vibration table and springs had a natural undamped resonance of 1.5Hz and the table was large enough to hold four force platforms (Figures 4.1A and 4.1B). The 1.5Hz resonance was damped out by plastic foam placed between the platform and the laboratory bench. Great care was taken to prevent unwanted vibrations travelling up connecting cables. In practice, this anti-vibration table removed all significant unwanted vibrations. As a result, while a stationary mouse was being monitored, its ventilatory frequency could be clearly observed in the force platform signal (Figure 4.4B). The whole system, set up for monitoring four individual cages, is shown in Figures 4.1A and 4.1B.

Procedure

Scratching was evoked using a range of known doses of pruritogens from previous studies; histamine (0.03-9.8 μ mol), HTMT (0.16 μ mol), imetit (3 μ mol) or clobenpropit (0.002-2.12 μ mol), by injecting the drugs intra-dermally (i.d.) into the back of the neck via a 26G needle. All drugs were dissolved in PBS (pH 7.4).

Individual mice received up to four injections and scratching was recorded both manually and by the automated detector on each occasion, to enable correlation and validation of the automated system being developed. Mice were placed one of the lightweight cages for 10 minutes conditioning prior to experimentation.

Scratching was recorded manually as described in chapter 2.1. In order to identify the individual number of scratch movements, mice were filmed for five-minute periods using a Sony digital video camera (model DCR-PC100E), and observed under slow motion.

Statistical Analysis

All data was analysed using GraphPad Prism (v3.02) software. Data are shown as a scatter plot and the goodness of fit is given as Pearson r-value.

4.3 Results

Response of Force Platform

In order to test that the force platform responded consistently in the frequency range we were interested in studying the frequency response of the force platform was examined by means of magnetic forces applied to a small permanent magnet fixed to the top of the platform. Opposing this magnet and secured to the bench was a coil which was driven from a constant current sine wave source. This generated a force proportional to the instantaneous current and allowed the frequency characteristics of the force platform to be investigated. The magnet and coil were calibrated against a digital balance to establish the relationship between a steady DC coil current and the resulting force on the magnet. At 15Hz the maximum sensitivity of the platform was about 500mV/gf but the system was normally used at a lower gain corresponding to about 150mV/gf.

The normalised frequency response of the force platform is shown in Figure 4.3. The platform responds consistently between the range of approximately 5 and 18Hz. Pilot studies had indicated that mice scratched at a frequency of 12 and 15Hz. Grooming behaviour, associated with the fore limbs generally occurs in the region of 8Hz. Although the platform responded in the frequency range related to scratching, to prevent the identification of grooming behaviour the repetitive event detector filters could be set at 8, 9, 10 or 11Hz (Low filter) to eliminate the grooming signal, with the upper filter limit being fixed at 20Hz.

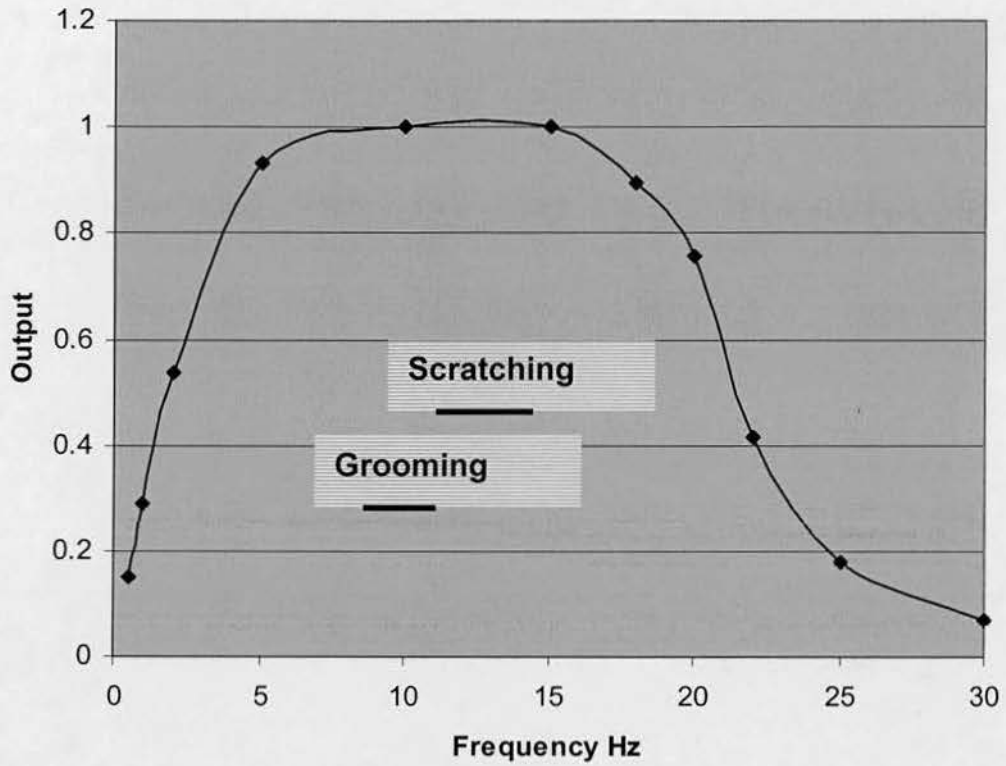


Figure 4.3 Normalised frequency response of force platform showing consistent responses around the 11-15 Hz frequency range which is associated with scratch movements. The labelled bars indicate the range of frequencies associated with scratching and with grooming.

Automated Measurement of Itch

Scratching was recorded as a Chart file (v4.22, ADInstruments); a sample trace is shown in figure 4.4A. The detector was set to record all repetitive activity of frequency greater than 11Hz (pilot studies had showed that below this level grooming behaviour was also recorded) and of amplitude greater than $\pm 125\text{mV}$. When such behaviour occurred, a staircase signal was produced, with each step in the output representing one scratch movement. This enabled us to measure both the 'bouts' of scratching evoked (3 or more scratch movements in one staircase) together with the total number of scratch movements per bout. Pilot studies indicated that the average number of scratch movements per bout was 5 ± 1 . At a frequency of approximately 12Hz, that indicated a bout of scratching lasts in the region of 0.4 seconds.

Correlation between 'bouts' of scratching detected manually and by automated system

The correlation between the numbers of bouts of scratching induced by histamine receptor agonists, as measured manually and by the automated detection system, is shown in Figure 4.5. The correlation was 0.91 (95% CI 0.88 – 0.93; Pearson r), showing that the automated detector consistently identified 91% of the bouts of scratching.

Correlation between total number of scratch movements detected manually and by automated system

The correlation between the total numbers of individual scratch movements induced by histamine receptor agonists, as assessed manually and by the automated detection system, is shown in Figure 4.6. There was a close relationship between the automated and manual counts, the Pearson r -value being 0.95 (95% CI 0.91 – 0.97), showing that the automated detector consistently identified 95% of the bouts of scratching.

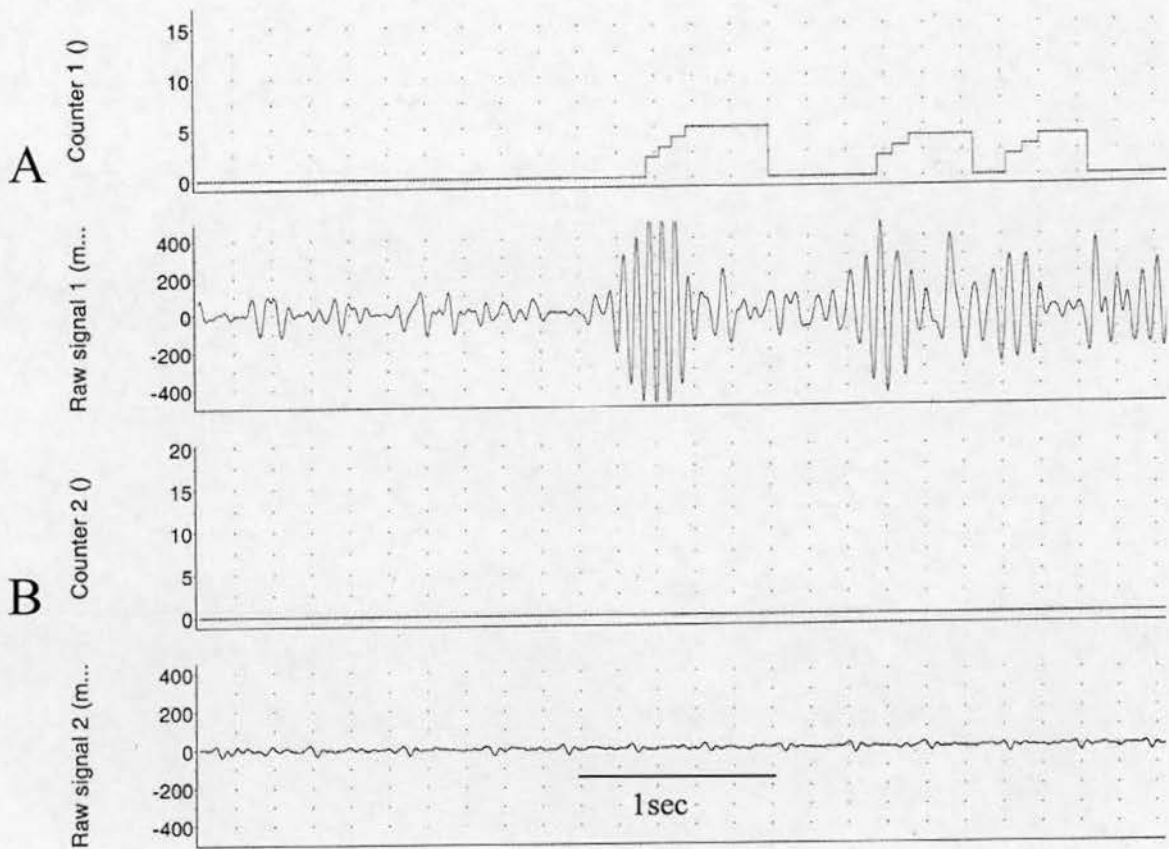


Figure 4.4 Sample trace from MacLab for two of the detection units. A: Two traces from one detection unit: lower part of the recording is the raw signal from the movement detector during scratching and the upper staircase is the processed signal representing a cumulative record of individual scratch movements evoked by injection of histamine ($3.3\mu\text{mol i.d.}$). The number of scratches in each of the three bouts in the panel is shown by the three ramps in the upper trace of the figure representing 5, 4 and 4 scratches consecutively. B: Output from a second detection unit following injection of 0.1 ml PBS: no activity was observed, either visually (from videotape) or in the output from the movement detector. Since the mouse did not move or scratch, there was no output from the scratch monitor channel (upper trace). The animal's breathing was detected as oscillations in the detector trace (frequency 180 breaths per minute), illustrating the high sensitivity of the detector system.

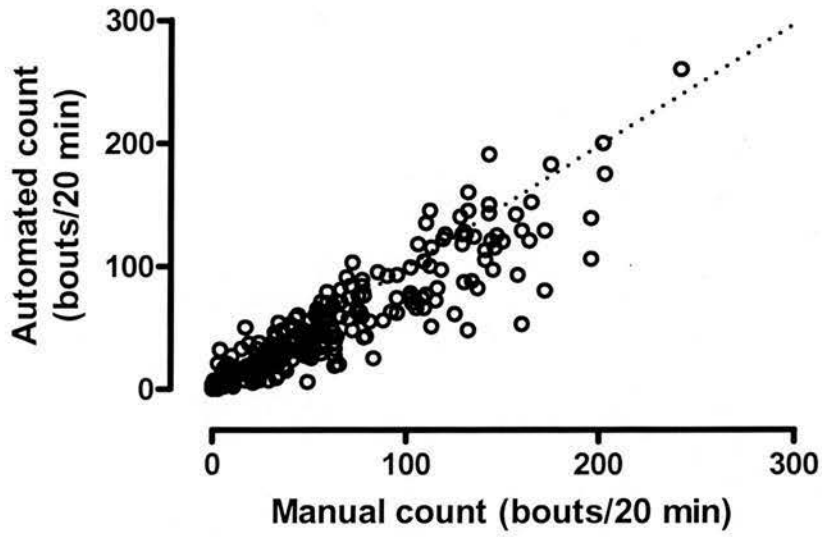


Figure 4.5 Plot showing the relationship between the numbers of bouts of scratching as recorded manually and by the automated detection system. The graph consists of 241 recordings made from 155 individual mice. The dashed line illustrates the line of equality.

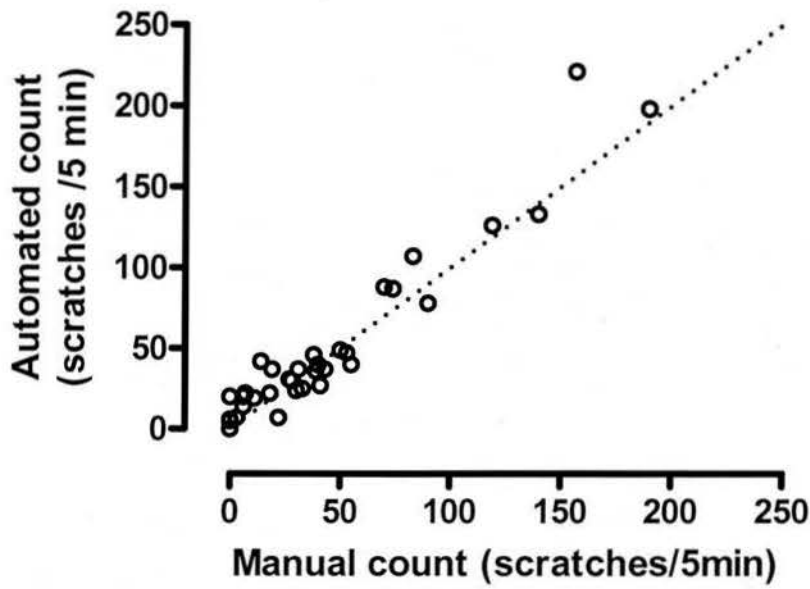


Figure 4.6 Graph showing relationship between the total number of scratch movements per bout as recorded manually from visual analysis during slow playback of videotaped recording, and by the automated detection system. The graph consists of 40 recordings made from 16 individual mice. The dashed line illustrates the line of equality.

4.4 Discussion

The findings from the present study provide evidence that the amount of scratching in a mouse can be measured automatically, based on characteristic repetitive movement of the hind paw. The system also reliably measures the total number of individual scratch movements of the hind paw over a given period of time.

Initial pilot studies using a simple strain gauge indicated that the pattern associated with scratching was distinct from general movement of the animal within the cage and also from other specific behaviours such as grooming. The pattern identified was the rhythmical beating activity seen during a bout of scratching, further investigation established the frequency of movement to be in the region of 12-15Hz. General movement created no regular frequency but there was a similar rhythmical pattern response to grooming behaviour. The majority of grooming behaviour could be eliminated using a filtering system as most grooming has a frequency lower than that of scratching at 8-10Hz.

The development of an effective automated detector of scratching is of potentially great benefit to researchers in the field of itch. Previously, scratching in mice had been identified by labour intensive manual observation and counting, either at the time, or at a later date through the use of a video camera and recorder. This is time consuming and highly unproductive, as few animals can be studied at any one time, not to mention highly tedious. This system allows the study of a number of mice at any one time as the rate-limiting step (aside from the number of detection systems in place), is the speed at which the investigator can place mice in the cages.

The final design of the detection system encompasses: a force platform that both holds the cage and encloses the strain gauge; a repetitive event detection unit containing the filtering system and a micro-controller to identify and record scratch movements; an anti-vibration table to remove unwanted background noise from the system and finally a PowerLab and PC to record and store the signals generated by

the system. The system currently consists of four force platforms and event detectors enabling the study of four mice at any one time.

Objective indicators of scratching in humans have also been developed. These systems generally rely on gross movement of the limbs rather than specific detectors of scratching (Felix & Shuster, 1975; Summerfield & Welch, 1980).

Two systems have previously been described in the literature to measure and record scratching in mice. Both systems use the repeated beating pattern of the hind limb as the indicator for scratching. The earlier system uses aluminium wire rings around the hind paw to elicit an electrical current in a ferrite rod and copper coil, surrounding the cage (Elliott *et al.*, 2000). Movement of the hind paw during scratching was reported to evoke a characteristic pattern of electrical current. The frequency of the scratching peaked at 15Hz, a figure similar to that identified during the present studies. The detector of scratching developed by Elliott *et al* identifies 80-85% of the scratching observed manually. However, the detector developed in this study is non-invasive to the mouse and does not require an aluminium ring around the hind limb, which would cause a loss of signal if the mouse managed to remove it. The most recently reported detector of scratching is very similar to that developed by Elliott *et al*. Again, the basis of the system relies on the generation of an electrical current identified by a metallic coil outside the cage, in this case the current is evoked by a magnet surgically inserted into the hind paw (Inagaki *et al.*, 2003). As with the detector developed in this study and that by Elliott *et al*, the system described by Inagaki *et al* identifies scratching based on frequency. Inagaki *et al* record activity of a frequency of 5-20Hz and can identify 90-95% of the scratching observed manually. This high correlation rate is achieved due to the wide range of frequencies measured. As the system only records activity from the hind paw, there is no signal overlap with the grooming behaviour (which primarily occurs using the fore paws). The obvious drawback with the system developed by Inagaki *et al*, is that despite the high correlation rate, the mice require prior surgery to insert the magnet into the hind paw.

The advantage the system described in the present studies has over both those previously presented in the literature, aside from the non-invasive nature, is that this detector is not simply a detector of hind limb activity but is a detector of, potentially, all activity, with the added capability of filtering out all other non-scratching behaviour. Therefore, leaving the ability, if required, to look at more than purely scratching.

The detection system enables users to learn more about the scratching behaviour of the mice than is possible from manual observation. As well as identifying bouts of scratching and the total number of scratch movements, the trace generated also gives an indication as to general activity, for example the trace can be analysed for the amount of time the mouse is active but not scratching. This may be of particular interest if drugs are being investigated with potential sedatory effects.

It would be feasible using a system very similar to that described to identify other repetitive behaviours in mice (such as grooming), or to expand the system for use in other small animal studies for which repetitive movements are a characteristic. Studies in rats or guinea pigs would be possible, however, the current system is designed to maximise the response generated in a 20-30g animal. A larger strain gauge would be required and it would be assumed that the frequency of scratching in a larger animal would be reduced compared to that in a mouse; pilot studies to determine the exact frequency of the behaviour would establish this.

The studies described in this section of the thesis have focused on the recording of acutely induced scratching. This system would be ideal for the recording of scratching in a chronically induced pruritic state. The labour intensity associated with manual observation of scratching over a longer period than 20 minutes would clearly be increased, whereas, this system could record scratching over periods of several hours/days.

In conclusion, the automated detector is a non-invasive and objective device for detecting scratching and could be used on a significantly larger scale in the study of pruritic and anti-pruritic drugs.

Chapter 5

Histamine-mediated Scratching in Mice

5.1 Introduction

Histamine is an endogenous amine, most commonly located in the brain, lungs, gut and skin of humans (Hill, 1990). Histamine is synthesised, stored and released peripherally from mast cells and to a lesser extent basophils. When released histamine acts on a family of receptors, known as histamine receptors. There are now recognised to be four histamine receptors; H_1 , H_2 , H_3 and H_4 (Leurs *et al.*, 2001). Each receptor subtype consists of a seven transmembrane domain protein, which is coupled to a G-protein secondary messenger system. Activation of each receptor by histamine activates a separate G-protein, which in turn activates a secondary messenger and evokes an intracellular cascade reaction, eliciting a cellular response (for example, nerve cell depolarization). H_1 receptor activation couples to a G_q protein that activates a secondary messenger known as phospho-inositol (PI). The other three histamine receptors all use cAMP as a secondary messenger, in the case of H_2 receptors there is up-regulation of this messenger, in the case of H_3 and H_4 receptors there is down-regulation (Haaksma *et al.*, 1990; Hill *et al.*, 1997).

Histamine has long been recognised as a mediator of itch in humans (Lewis, 1927). Intradermal administration of histamine can elicit itch in doses as low as 0.7nmol (Hagermark, 1973). Although traditional H_1 receptor antagonists are effective at reducing histamine-induced itch (Hagermark *et al.*, 1979) and are widely used clinically for treating pruritus (Greaves, 1997), they are not in fact effective antipruritics in clinical conditions such as atopic eczema (Yosipovitch *et al.*, 2003). It has been claimed that the effectiveness of H_1 receptor antagonists at relieving itch results from central sedation rather than peripheral actions on sensory nerves, in conditions where histamine is not seen as the primary mediator (Krause & Shuster, 1983; Savin, 1980; Savin *et al.*, 1986). Histamine H_2 receptor antagonists are also used clinically for treating itch (Monroe *et al.*, 1981; Ring & Thomas, 1989), but their ability to inhibit itching is not conclusive (Davies & Greaves, 1980; Hagermark *et al.*, 1979). Overall the evidence suggests that mechanisms other than those involving H_1/H_2 receptors are involved in itch.

Until recently it was commonly believed that histamine-induced itch was well understood and that, although an important mediator in some conditions, histamine could not be responsible for all clinical pruritic states, due to the lack of effectiveness of H₁ and H₂ receptor antagonists in treating these conditions. The discovery of two new histamine receptors has, however, opened up the possibility that histamine may be operating through a previously unidentified receptor mechanism, to evoke itch. Histamine could elicit itch through an action on either the H₃ receptor or the H₄ receptor. The H₃ receptor is localized to nervous tissue, mainly in the brain (Arrang *et al.*, 1983; Haaksma *et al.*, 1990; Lovenberg *et al.*, 1999). It has been postulated that there are several potential isoforms of the H₃ receptor (Coge *et al.*, 2001), which are believed to respond differently to agonists and antagonists, thus making the characterization of these receptors very difficult. Mouse and rat H₃ receptors have been identified, but, whether different isoforms exist in these species is at present unknown (Leurs *et al.*, 2001; Lovenberg *et al.*, 2000). The most recently described histamine receptor is the H₄ receptor (Liu *et al.*, 2001a; Nakamura *et al.*, 2000; Nguyen *et al.*, 2001; Oda *et al.*, 2000; Zhu *et al.*, 2001). Unlike the H₃ receptor, the H₄ receptor has not been identified in the brain but appears to be associated with tissues involved in the immune response. The H₄ receptor is commonly localized to bone marrow and eosinophils/leukocytes (Liu *et al.*, 2001a; Oda *et al.*, 2000). The H₄ receptor has also been shown to play a role in mast cell chemotaxis, indicating its potential as a mediator of inflammatory and possibly pruritic responses (Hofstra *et al.*, 2003; O'Reilly *et al.*, 2002).

In murine studies itch is estimated by measuring the scratching elicited by itch-provoking agents (Kuraishi *et al.*, 1995), as described in Chapter 3. Although some reports have questioned the potential role of histamine as a pruritogen in mice (Kuraishi *et al.*, 1995), others have shown that histamine H₁ antagonists are effective at reducing histamine-induced scratching in this species (Inagaki *et al.*, 1999; Sugimoto *et al.*, 1998). When examining histamine-mediated scratching in mice (and other animal models) it is necessary to be aware of both strain differences (Inagaki *et al.*, 2001) and also possible species differences between humans and mice. Differences between strains of mice have previously been discussed in chapter 3,

however, species differences also exist. Recent evidence suggests that H₄ receptors in different species have differing affinities for histamine; it is reported that histamine has an eight fold lower affinity for murine H₄ receptors than for human or guinea pig H₄ receptors (Liu *et al.*, 2001b). The H₁ receptor has also been shown to have species-dependent differences; some agonists and antagonists have different affinities for human and guinea pig H₁ receptors (Seifert *et al.*, 2003).

The use of receptor selective agonists and antagonists as tools for characterizing receptor mechanisms is useful, but can be flawed if incorrect assumptions are made about the activity of these drugs at different receptors. As has been described above, there appear to be several isoforms of the human H₃ receptor, which respond differently to various agonists and antagonists (Coge *et al.*, 2001). No evidence exists in mice to support this finding; however, until a complete characterization of these receptors and the drugs that act upon them has been established it is important not to misinterpret results. The action of drugs known to act at the well characterized H₁ and H₂ receptors may also have actions at the newly discovered H₃ and H₄ receptors. For example the commonly used H₁ receptor antagonist, mepyramine, has the same affinity for the human H₄ receptor as does histamine (Nguyen *et al.*, 2001).

This study was undertaken to investigate the role of H₁, H₂, H₃ and H₄ receptors in histamine-induced itch, primarily in BalbC mice, based on the quantification of acutely induced scratching.

5.2 Materials & Methods

Procedure

Details on the induction of itch and the measurement of scratching are described in chapter 2.1. Experiments were conducted in a total of 265 female BalbC mice.

In experiments involving antagonists, scratching was induced by a mid-range dose of histamine, which was repeated 30 and again 90 minutes after treatment with either an

H₁, H₂ or H₃/H₄ antagonist (20mgkg⁻¹ i.p.). This dose of antagonist was selected on the basis of its effectiveness in reducing the scratching evoked by receptor selective H₁, H₂ or H₃ agonists.

The effect of antagonists on receptor-selective agonist-induced scratching were studied in separate groups of mice, as desensitization to the agonists precluded repeated dosing. The responses to agonist was recorded 30 minutes after antagonist treatment and compared to responses obtained from vehicle (PBS) treated mice. The dose of agonist was selected on the ability of that dose to elicit scratching in each mouse studied, with the aim of eliciting approximately 50% of the maximum scratching response.

As described in Chapter 2.4 non-parametric statistical analyses were used. The mean 'apparent' ED₅₀ was calculated from pooled data and was the dose required to elicit half the apparent maximal response.

5.3 Results

Histamine-induced scratching in BalbC mice

Histamine caused dose-dependent scratching in all the BalbC mice studied, as previously described in Chapter 3.3. Scratching was not evoked at lower doses (below 0.8μmol), but was consistently observed with higher doses, reaching a maximum at 26μmol histamine. Above this dose there was no further increase, and often a decrease, in scratching. Histamine-induced scratching reached a maximum of 73±12 bouts (mean±sem, n=8), with a mean apparent ED₅₀ of 5.8μmol.

Scratching induced by phosphate buffered saline served as a control (5±1 bouts of scratching, n=12), as previously summarised in Chapter 3.3.

Itch inducing effects of an intradermally administered H₁ receptor antagonist, mepyramine, in BalbC mice

During pilot studies mepyramine was administered intradermally, to the area around the back of the neck. Mepyramine ($0.1\text{--}1\text{mgkg}^{-1}$) did not reduce histamine evoked scratching: histamine ($8\mu\text{mol}$, i.d.) evoked 42 ± 3 bouts of scratching (median=41, $n=8$); 30 minutes following mepyramine treatment (0.1mgkg^{-1} , i.d.), histamine ($8\mu\text{mol}$, i.d.) evoked 49 ± 8 bouts of scratching (median=52, $n=6$); 30 minutes following a higher dose of mepyramine (1mgkg^{-1} , i.d.), histamine ($8\mu\text{mol}$, i.d.) evoked 55 ± 6 bouts of scratching (median=54, $n=6$).

Higher doses of mepyramine, administered intradermally evoked a scratching response independent of histamine injection. Mepyramine (10mgkg^{-1} i.d.) induced 35 ± 11 bouts of scratching ($n=4$). Due to the unexpected itch inducing properties of mepyramine, future experiments focused on the systemic administration of antagonists, which did not themselves evoke a scratching response.

Itch inducing effects of intradermally administered H₂ and H_{3/4} receptor antagonists in BalbC mice

Unlike mepyramine, cimetidine (H₂ receptor antagonist) and thioperamide (H₃ and H₄ receptor antagonist), were relatively ineffective at inducing scratching. Cimetidine (10mgkg^{-1} , i.d. $n=11$) evoked 6 ± 2 bouts of scratching (median=4), whereas thioperamide (10mgkg^{-1} , i.d. $n=6$) evoked 14 ± 5 bouts of scratching (median=12). Neither of these responses differs significantly from scratching evoked by PBS ($P>0.05$, Mann Whitney test). Despite these drugs not eliciting scratching when administered intradermally, further experiments using these compounds were conducted using systemic administration in order to allow direct comparison with studies using mepyramine, which did elicit a scratching response when administered intradermally (see above).

Role of histamine H₁ receptors in histamine-induced scratching in BalbC mice

The selective histamine H₁ receptor agonist, HTMT, evoked dose dependent scratching in all six mice studied (Figure 5.1A). Maximal scratching was 133±17 bouts, with a mean apparent ED₅₀ for HTMT of 0.1µmol.

Histamine-induced (8µmol, i.d.) scratching was significantly reduced following mepyramine (20mgkg⁻¹, i.p. $P<0.05$, n=6, Figure 5.2Ai). PBS, used as a control for the antagonist, did not significantly reduce histamine-induced scratching ($P>0.05$, n=5, Figure 5.2Aii).

HTMT-induced scratching (0.2µmol, i.d.) was significantly reduced in mice pre-treated with mepyramine (20mgkg⁻¹, i.p.) in comparison with vehicle treated mice ($P<0.05$, Figure 5.2B). Lower doses of mepyramine (1-10mg/kg⁻¹) did not significantly reduce HTMT-induced scratching.

HTMT-induced scratching (0.2µmol, i.d.) was also significantly reduced by pre-treatment with the non-sedating histamine H₁ receptor antagonist, terfenadine (20mgkg⁻¹, i.p.) as compared with vehicle treated mice ($P<0.05$, Figure 5.2C). Lower doses of terfenadine (1-10mgkg⁻¹) did not significantly reduce HTMT-induced scratching ($P>0.05$) but there was variability with responses, and the trend indicated that the anti-scratch effect of terfenadine was dose related.

Role of histamine H₂ receptors in histamine-induced scratching in BalbC mice

Dimaprit, histamine H₂ receptor agonist, did not induce dose dependent scratching in mice (0.04-40µmol, i.d. n=3, Figure 5.1D).

The H₂ receptor antagonist cimetidine (20mgkg⁻¹, i.p.) did not significantly reduce histamine-induced scratching ($P>0.05$, n=6, Figure 5.3).

Role of histamine H₃ receptors in histamine-induced scratching in BalbC mice

Imetit, histamine H₃ and H₄ receptor agonist, induced dose-dependent scratching in all six mice studied (Figure 5.1C). The maximal level of scratching was 69±23 bouts, with a mean apparent ED₅₀ for imetit of 0.9µmol.

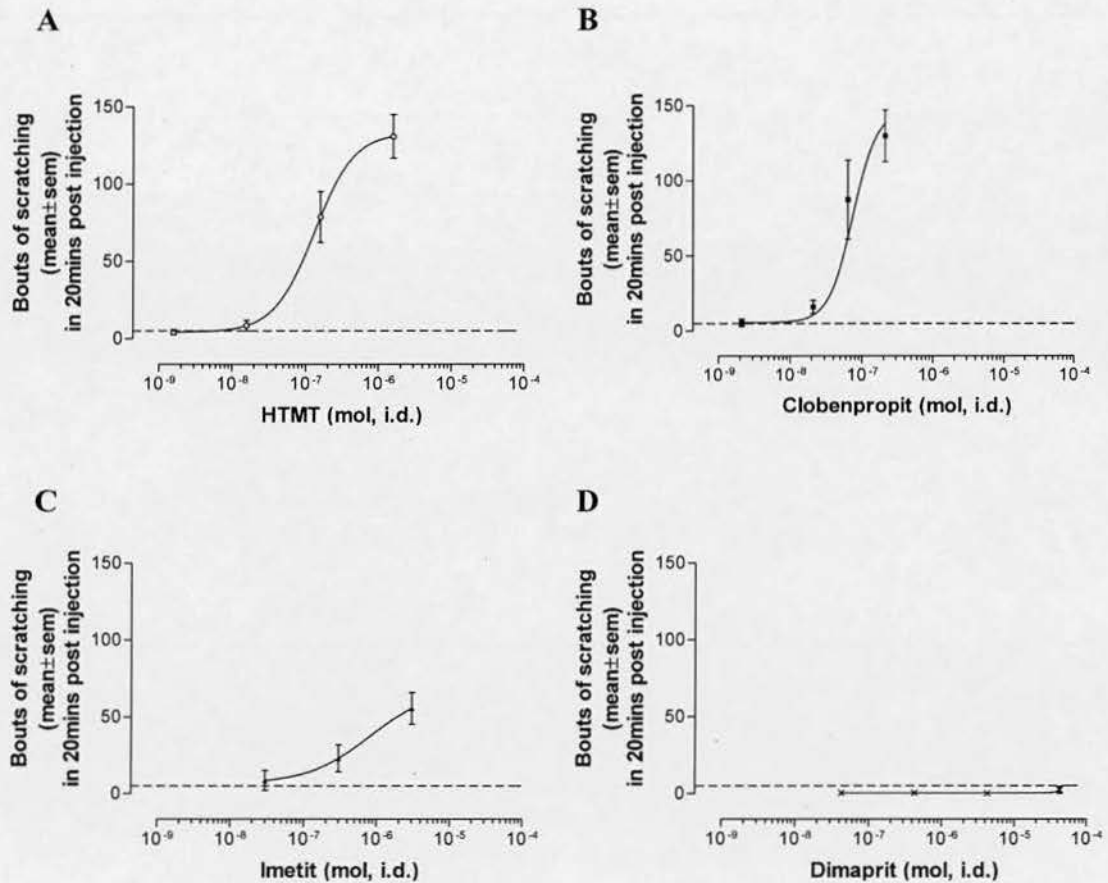


Figure 5.1 Pooled data illustrating scratching induced by (A) HTMT (H_1 receptor agonist, $n=6$), (B) clobenpropit (H_4 agonist, $n=10$), (C) imetit (H_3/H_4 agonist, $n=6$) and (D) dimaprit (H_2 agonist, $n=3$) in BalbC mice. HTMT, clobenpropit, and to a lesser extent imetit, caused dose related scratching, whereas dimaprit had no effect. (Dashed line illustrates PBS induced scratching).

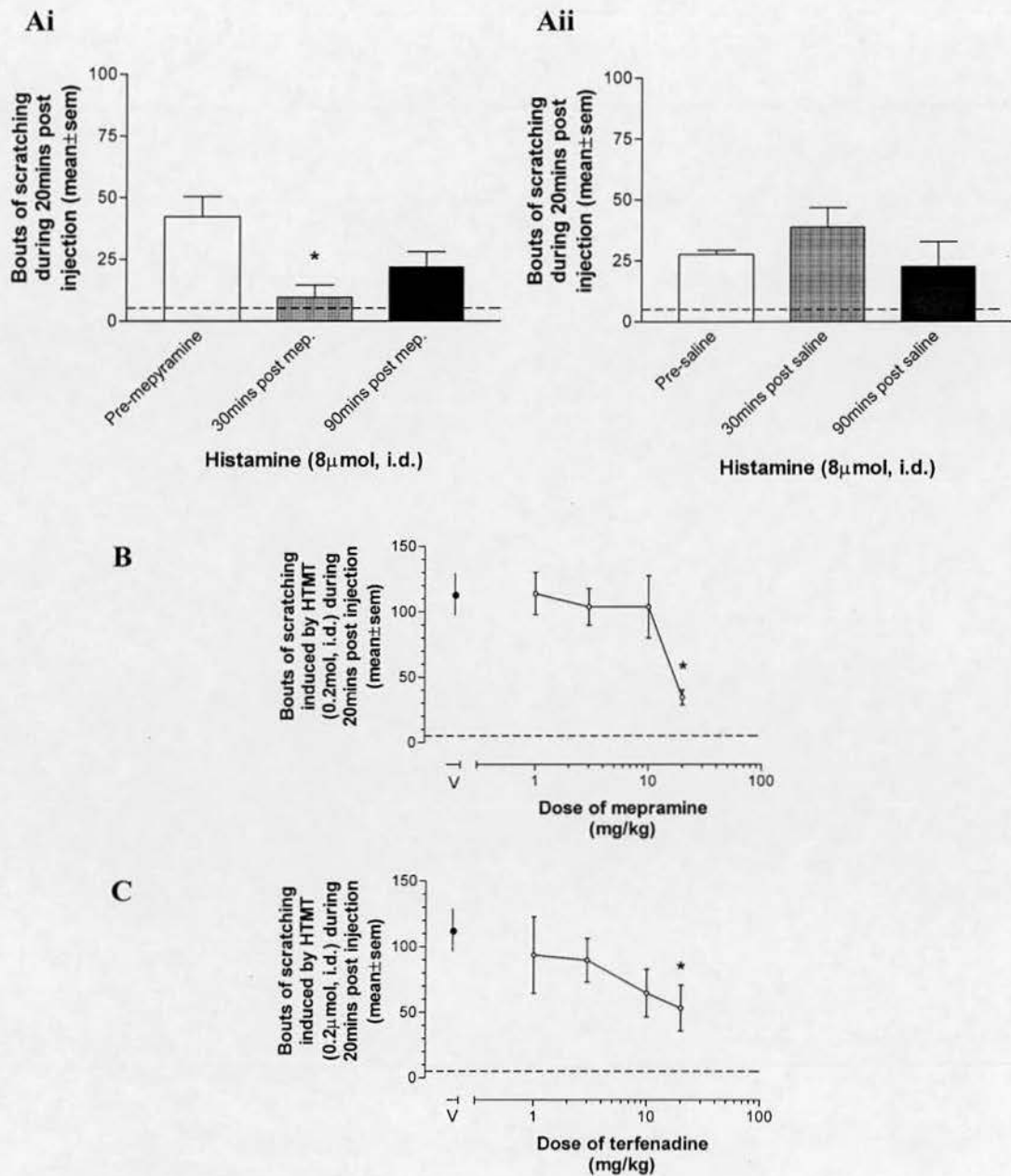


Figure 5.2 (Ai) Mepyramine (20mgkg^{-1} , i.p.) significantly reduced scratching induced by histamine ($8\mu\text{mol}$, i.d.) in female BalbC mice, 30 minutes after treatment with the antagonist versus pre-treatment value ($*P<0.05$, Wilcoxon matched pairs test, $n=6$). (Aii) Saline vehicle did not significantly reduce scratching induced by histamine ($P>0.05$, Wilcoxon matched pairs test, $n=5$). (B) Mepyramine (20mgkg^{-1} , i.p.) pre-treated mice showed a significantly lower scratching response to HTMT ($0.2\mu\text{mol}$, i.d.) in comparison with vehicle treated animals ($*P<0.05$, Mann Whitney test, $n=6$ in each group). Mepyramine at lower doses (1mgkg^{-1} , 3mgkg^{-1} or 10mgkg^{-1}) did not significantly reduce HTMT-induced scratching ($P>0.05$, Mann Whitney test, $n=6$ in each group). (C) Terfenadine significantly reduced scratching induced by HTMT ($0.2\mu\text{mol}$, i.d.) in a dose dependent manner (terfenadine 20mgkg^{-1} versus control, $*P<0.05$, Mann Whitney test, $n=6$ in each group). (Dashed line illustrates PBS induced scratching).

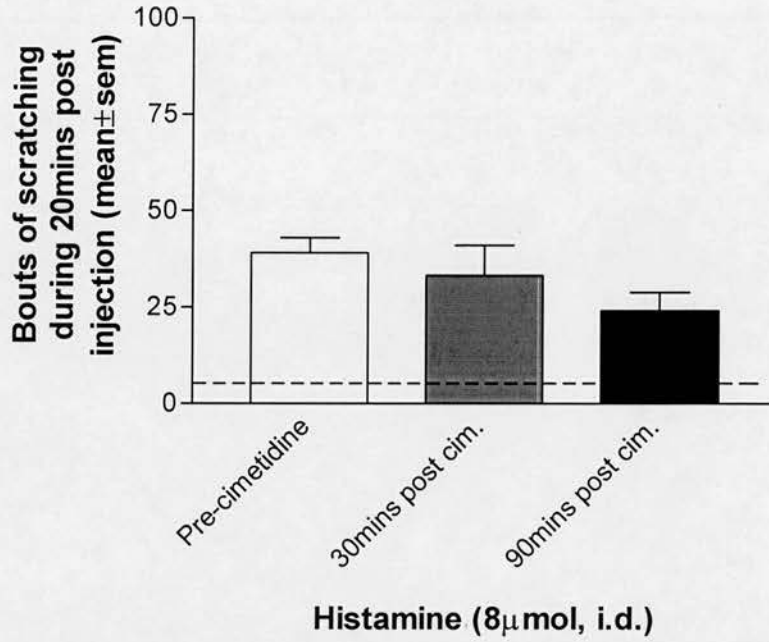


Figure 5.3 Cimetidine (20mgkg⁻¹, i.p.) did not reduce histamine-induced scratching in BalbC mice (8 μ mol, i.d. $P > 0.05$, Wilcoxon matched pairs test, $n = 6$). (Dashed line illustrates PBS induced scratching).

Scratching induced by histamine ($3\mu\text{mol}$, i.d.) was significantly reduced following treatment with the dual H_3 and H_4 receptor antagonist thioperamide (20mgkg^{-1} , i.p. $P<0.05$, $n=6$, Figure 5.4Ai). PBS vehicle (i.p.) did not significantly reduce histamine-induced scratching ($P>0.05$, $n=6$, Figure 5.4Aii).

Imetit-induced scratching ($3\mu\text{mol}$, i.d.) was significantly reduced in mice pre-treated with thioperamide (20mgkg^{-1} , i.p.) in comparison with vehicle treated animals ($P<0.05$, Figure 5.4B). Lower doses of thioperamide ($1\text{-}10\text{mgkg}^{-1}$) reduced the mean level of imetit-induced scratching, but due to variability in responses, the difference between means (versus PBS) was not statistically significant ($P>0.05$).

Studies conducted using a different H_3 receptor antagonist (RWJ-662733, RW Johnson), confirmed histamine evoked scratching was significantly reduced by an H_3 receptor antagonist. Histamine ($8\mu\text{mol}$, i.d.) evoked of 33 ± 2 bouts of scratching prior to antagonist treatment, this was reduced to 7 ± 3 bouts 30 minutes following antagonist treatment ($P<0.05$, Paired t-test, $n=4$).

Role of histamine H_4 receptors in histamine-induced scratching in BalbC mice

Clobenpropit, a histamine H_4 receptor agonist and H_3 receptor antagonist, induced dose-dependent scratching in all ten mice studied (Figure 5.1B). The maximal level of scratching was 144 ± 20 bouts, with a mean apparent ED_{50} for clobenpropit of $0.05\mu\text{mol}$.

Clobenpropit-induced scratching ($0.06\mu\text{mol}$, i.d.) was significantly lower in mice pre-treated with the highest two doses of thioperamide used (10mgkg^{-1} and 20mgkg^{-1} , i.p.) in comparison with vehicle treated mice ($P<0.05$, Figure 5.5).

H_1 versus H_3/H_4 receptor involvement in itch in BalbC mice

H_1 agonist, HTMT ($0.2\mu\text{mol}$, i.d.), induced scratching was not significantly reduced by the H_3/H_4 receptor antagonist, thioperamide (20mgkg^{-1} , Figure 5.6A, $P>0.05$ versus vehicle), whereas it was by the H_1 receptor antagonists, terfenadine and mepyramine (as described above).

The H_4 agonist, clobenpropit ($0.06\mu\text{mol}$, i.d.) induced scratching was not significantly reduced by mepyramine (sedatory), or terfenadine (non-sedatory), H_1

receptor antagonists (20mgkg^{-1} , Figure 5.6B, $P>0.05$ versus vehicle), whereas it was by the H_3/H_4 receptor antagonist thioperamide (see above).

Effects of histamine receptor antagonism in other strains of mice

The effects of mepyramine, H_1 antagonist, and thioperamide, H_3/H_4 antagonist, on histamine-induced scratching were investigated in ICR and C57/Bl6 strains of mice. These strains, as previously described in chapter 3.3, are responsive to lower doses of histamine than the BalbC strain. Studies into the antagonistic effects of mepyramine and thioperamide in these strains did not provide clear evidence due to large variability in the responses to histamine. There was no particular dose of histamine that consistently evoked a scratching response, and repeated doses of histamine evoked largely variable responses. For this reason, studies into the anti-pruritic effects of mepyramine and thioperamide were not continued in these strains.

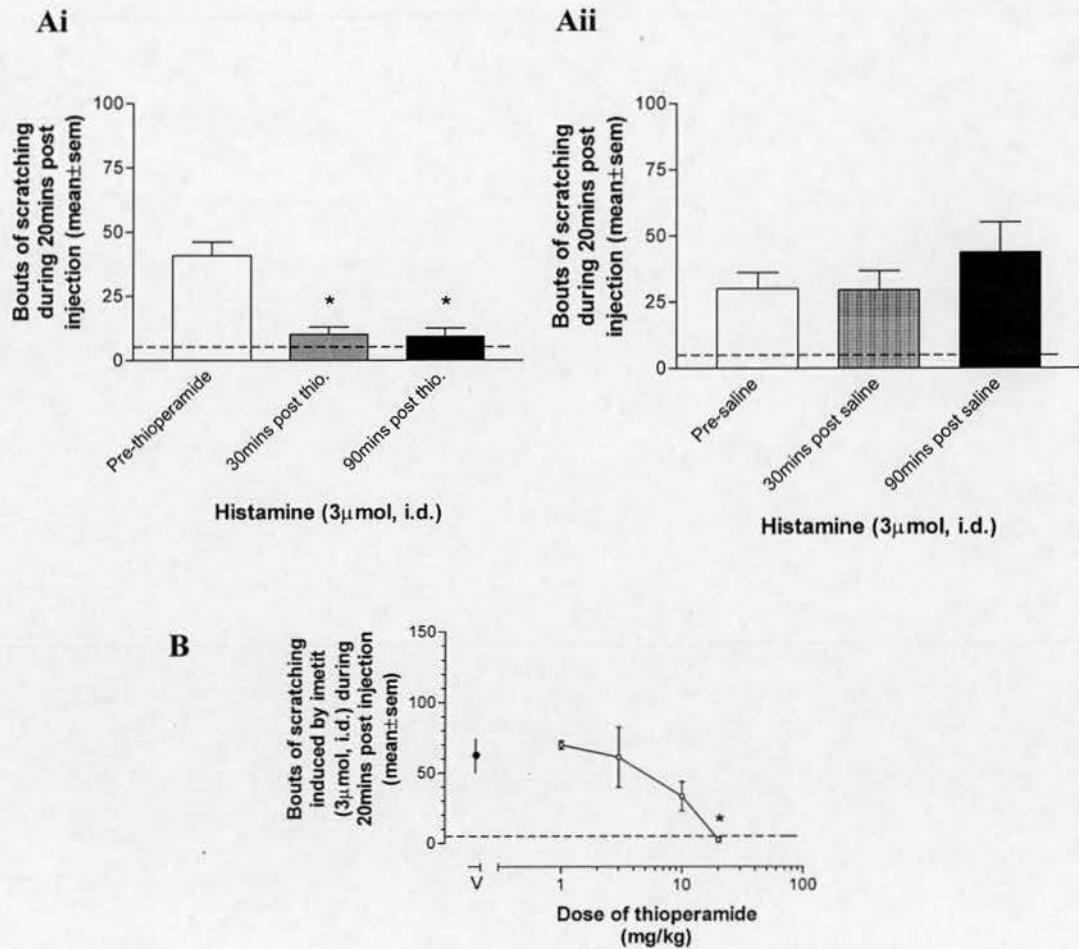


Figure 5.4 (A) Thioperamide (20mgkg^{-1} , i.p.) significantly reduced scratching induced by histamine in BalbC mice ($3\mu\text{mol}$, $*P<0.05$, Wilcoxon matched pairs test, $n=6$). (Aii) Saline vehicle did not significantly reduce scratching induced by histamine ($P>0.05$, Wilcoxon matched pairs test, $n=6$). (B) Thioperamide (20mgkg^{-1} , i.p.) pre-treated mice showed a significantly lower scratching response to imetit ($3\mu\text{mol}$, i.d.) in comparison with vehicle treated animals ($*P<0.05$, Mann Whitney test, $n=5$ in each group). Thioperamide at lower doses (1mgkg^{-1} , 3mgkg^{-1} or 10mgkg^{-1}) did not significantly reduce imetit-induced scratching ($P>0.05$, Mann Whitney test, $n=4$ in each group). (Dashed line illustrates PBS induced scratching).

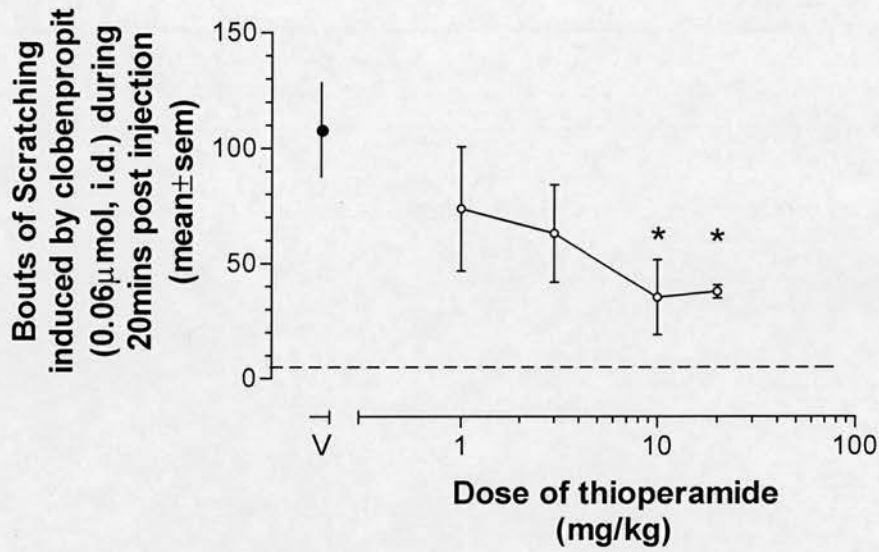


Figure 5.5 Thioperamide (20mgkg^{-1} and 10mgkg^{-1} , i.p.) pre-treated BalbC mice showed a significantly lower scratching response to clobenpropit ($0.06\mu\text{mol}$, i.d.) in comparison with vehicle treated animals (* $P < 0.05$, Mann Whitney test, $n = 6$ in each group). Thioperamide at lower doses (1mgkg^{-1} or 3mgkg^{-1}) did not significantly reduce imetit-induced scratching ($P > 0.05$, Mann Whitney test, $n = 6$ in each group). (Dashed line illustrates PBS induced scratching).

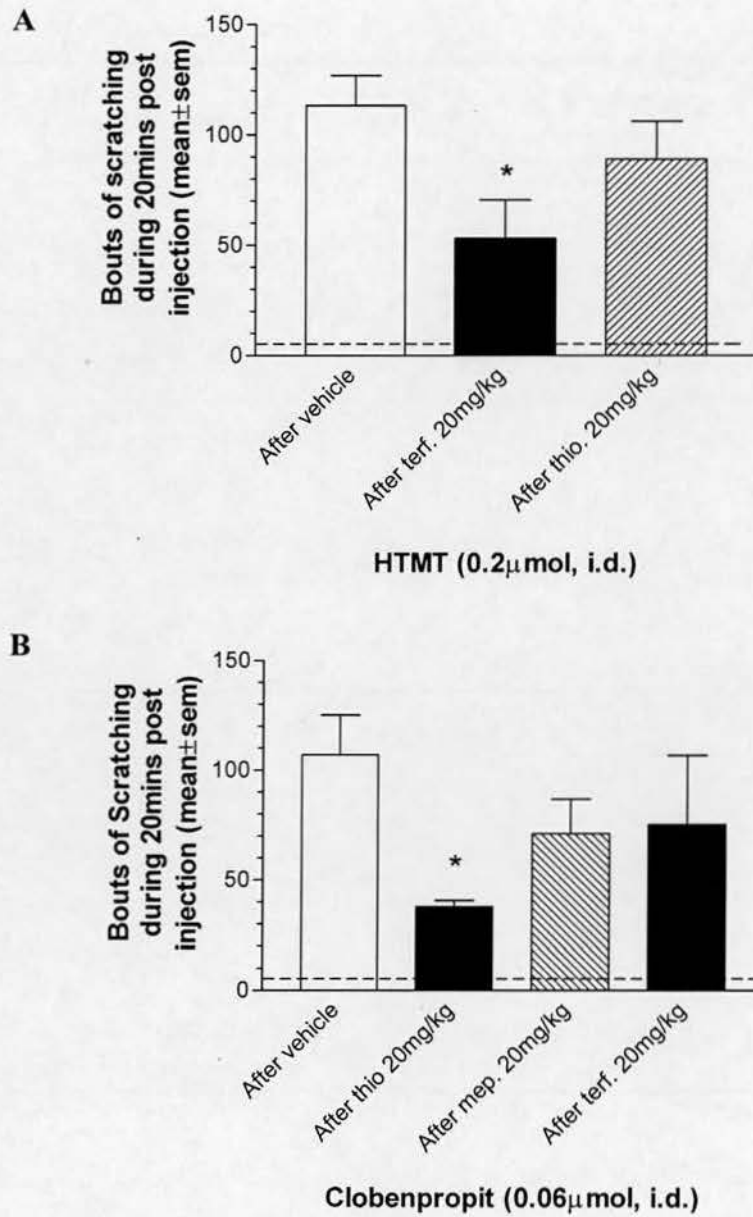


Figure 5.6 (A) Thioperamide (20mgkg^{-1} , i.p.) did not significantly reduce HTMT-induced scratching ($0.2\mu\text{mol}$, i.d.) in comparison with vehicle treated mice ($P>0.05$, Mann Whitney test, $n=6$ in each group), whereas the H_1 antagonist, terfenadine (20mgkg^{-1} , i.p.) reduced HTMT-induced scratching ($*P<0.05$, Mann Whitney test, $n=6$ in each group). (B) Mepyramine and terfenadine, sedatory and non-sedatory H_1 receptor antagonists respectively (20mgkg^{-1} , i.p.) did not significantly reduce clobenpropit induced scratching ($0.06\mu\text{mol}$, i.d.) in comparison to vehicle treated mice ($P>0.05$, Mann Whitney test, $n=6$ in each group), whereas the $H_{3/4}$ antagonist, thioperamide (20mgkg^{-1} , i.p.) did reduce clobenpropit-induced scratching ($*P<0.05$, Mann Whitney test, $n=6$ in each group). (Dashed line illustrates PBS induced scratching).

5.4 Discussion

Results from studies in this section of the project provide evidence for the involvement of histamine H_4 receptors in the scratching (itch) evoked by histamine, in mice. The lack of effectiveness of classical H_1 -receptor antihistamines in alleviating many chronic pruritic conditions (Greaves, 1997) led to the focus of research for anti-pruritic drugs being shifted from histamine to other putative mediators. This study suggests that histamine can generate itching in BalbC mice via the recently discovered H_4 and (possibly) H_3 receptors.

It was confirmed that acutely administered histamine induces dose-dependent scratching when injected intradermally in BalbC mice (Inagaki *et al.*, 2001), as it does in humans (Keele & Armstrong, 1964). It has previously been shown in mice that the dose of histamine required to induce scratching in this species is substantially higher (approximately 100 times) than that which evokes itch in humans (Inagaki *et al.*, 2001; Kuraishi *et al.*, 1995; Maekawa *et al.*, 2000). This may reflect differences in the sensitivity of histamine receptors to agonists, in mice and man; such evidence has been shown to exist between human and guinea pig H_1 receptors (Liu *et al.*, 2001b) and also between H_4 receptors of humans and mice (Seifert *et al.*, 2003). But also illustrates that comparisons of findings made in mouse and man need to be interpreted cautiously.

Early pilot studies provided an unanticipated problem with the peripherally administered H_1 receptor antagonist, mepyramine. The antagonist itself was capable of evoking scratching when injected i.d., and although the other antagonists used did not evoke scratching it was decided to administer antagonists systemically in all cases to avoid further similar complications. A suggestion as to why this scratching response to mepyramine may have occurred is described below.

The H_1 antagonist, mepyramine reduced histamine-induced scratching, as previously established in mice (Sugimoto *et al.*, 1998) and humans (Hagermark *et al.*, 1979). Some authors have suggested this may, at least in part, be due to the sedating

properties of H₁ antagonists rather than any peripheral action at pruritoceptors (Krause & Shuster, 1983). We have shown that injection of the histamine H₁ receptor agonist HTMT induces acute scratching in mice, strongly suggesting the involvement of peripheral H₁ receptors in itch. This action of HTMT was shown to be mediated via H₁ receptors because mepyramine reduced agonist-induced scratching. Mepyramine is a traditional H₁ receptor antihistamine, with an affinity 1000 times greater at the H₁ receptor than at the H₂ or H₃ receptor, see below for actions of mepyramine at H₄ receptor, however, mepyramine also has central sedating actions (Rang *et al.*, 1996). In order to prove the role of an H₁ mechanism we used a second H₁ receptor antagonist, which lacked central actions. This study showed that the non-sedating H₁ receptor antagonist, terfenadine, significantly reduced HTMT-induced scratching, thus proving that a sedatory effect is not the sole action of H₁ receptor antihistamines.

This study has found no evidence for involvement of H₂ receptors in the scratching evoked by histamine in mice. The H₂ receptor agonist, dimaprit, did not evoke scratching, and the H₂ receptor antagonist, cimetidine, had no significant effect on histamine-induced scratching. This finding is consistent with evidence in the literature: although a combination of H₁ and H₂ receptor antagonists is sometimes used clinically for treating itch (Drake *et al.*, 1994; Ring *et al.*, 1999), H₂ receptors are not thought to be crucial in histamine-evoked itch (Davies & Greaves, 1980; Hagermark *et al.*, 1979).

The discovery of histamine H₃ (Lovenberg *et al.*, 1999) and, more recently, H₄ (Oda *et al.*, 2000) receptors, has implications for the involvement of histamine as a mediator of itch (Repka-Ramirez, 2003). The tissue distribution of both receptors indicates a potential role for them in the periphery: the H₃ receptor affects histamine-mediated axonal transport in mouse dorsal root ganglia (Amano *et al.*, 2001) and is localized to nervous tissue (Lovenberg *et al.*, 1999), whereas the H₄ receptor is associated with immune responses, is preferentially expressed on leukocytes (Oda *et al.*, 2000), and plays a role in mast cell chemotaxis (Hofstra *et al.*, 2003). The present study found that the dual H₃ and H₄ receptor antagonist thioperamide (greater than

1000 times more selective for the H₃ and H₄ receptors than H₁ or H₂ receptors) significantly reduced histamine-induced scratching in mice. A second H₃ receptor antagonist, RWJ-662733 from RW Johnson, also reduced histamine evoked scratching, although this cannot discount any action this compound may also have on H₄ receptors. The H₃ and H₄ receptor agonist, imetit, evoked scratching that was reduced by thioperamide. In order to try and differentiate histamine H₃ from H₄ mechanisms the compound clobenpropit was used, which is an H₃ antagonist, but an H₄ agonist (Oda *et al.*, 2000). Clobenpropit induced dose-dependent scratching in mice, which was antagonised by thioperamide. This finding implicates a peripheral H₄ receptor in scratching induced by intradermal histamine.

It can be concluded that distinct H₁ and H₄ receptor mechanisms are responsible for itching evoked by exogenous histamine, and this is supported by the data that shows H₁-agonist induced scratching was not significantly reduced by the H₃/H₄ antagonist. Also, scratching evoked by an H₄ agonist was not significantly reduced by either the sedatory or non-sedatory H₁ receptor antagonists.

Although this data strongly suggests a role for H₁ and H₄ receptors in histamine mediated scratching, it is not possible to determine whether H₃ receptors play a role. The difficulty is that imetit, thioperamide and clobenpropit act at multiple histamine receptor subtypes. The H₃/H₄ receptor agonist imetit induced less scratching than the H₁ and H₄ agonists, which could be because imetit has a low affinity for the murine H₄ receptor, or it may be due to a combination of H₃ and H₄ actions. It has recently been reported that mepyramine binds to the H₄ receptor with a similar affinity to histamine; this may explain why H₁ anti-histamines relieve some clinical itch conditions, even if H₁ receptors are not involved (Nguyen *et al.*, 2001). This may also explain the possible itch evoking properties of intradermally administered mepyramine, as described earlier. Further studies using drugs with greater selectivity for histamine receptor subtypes, particularly selective H₄ receptor agonists and antagonists, are required to distinguish between an H₃ and/or an H₄ mechanism, but based on the use of currently available drugs, it is possible to conclude that H₄ as well as H₁ receptors contribute to itch evoked by histamine in BalbC mice. Based on

the tissue distribution of histamine H₄ receptors in immune response tissue (Liu *et al.*, 2001a; Oda *et al.*, 2000) it is realistic to propose that H₄ mediated itch may be elicited through a pathway involving eosinophil infiltration to inflamed or damaged areas of skin. Further research would be required to determine whether there is a scientific basis to this suggestion.

Pilot studies into the mechanism of action of histamine-evoked scratching in ICR and C57/Bl6 mice proved inconclusive. These strains are more sensitive to histamine, as described in chapter 3, by approximately 30 times. This sensitivity lead to very variable responses, unlike those seen in the BalbC strain. This variability of responses meant that we were unable to produce clear evidence concerning the effects of agonists and antagonists selective for the different types of histamine receptor. It would be fascinating to determine whether this increased sensitivity to histamine is due to different receptor subtypes being present, different isoforms being expressed that respond to drugs in a distinct manner or simply that different behavioural responses to drugs are evoked between strains. Establishing a greater understanding of the reasons for the differences in histamine response between strains may also provide us with clues as to differences in histamine responses between humans and mice.

This evidence is unable to provide definitive evidence as to whether the responses to histamine that was being studied result from direct actions on pruritoceptors (the most likely explanation), or indirect actions via mast cells, basophils etc (Poli *et al.*, 1994; Ring & Thomas, 1989). Nevertheless, the results from these functional studies provide sufficient evidence to warrant further investigation on the involvement of H₄ receptors in the physiology and pathophysiology of itch in both mouse and man, and may be sufficient to target H₄ receptor antagonists for developing specific anti-itch drugs.

Chapter 6

Alternative Mediators of Scratching in mice

6.1 Introduction

Despite the role of histamine as a mediator of itch, as described in chapter 5, it is by no means the only itch inducing substance that requires investigation. Histamine was presumed to be the most significant mediator of itch in clinical human conditions since its discovery as a provoker of pruritus (Lewis, 1927). Two main pieces of evidence exist that support a role for other mediators of the sensation, aside from histamine. The first evidence indicating that histamine is not the only mediator of itch comes from the ineffectiveness of traditional histamine H₁ receptor antihistamines at relieving itch in a number of clinical conditions such as atopic eczema (Yosipovitch *et al.*, 2003). Clearly, if histamine were responsible for the itch associated with such conditions we would expect such drugs to alleviate the itch of all clinically pruritic states. Secondly, experimentally induced itch, using histamine, indicates that responses to histamine are reduced after repeated application, tachyphylaxis (Stahle-Backdahl *et al.*, 1988). If histamine alone were to mediate clinical itch, we would expect a gradual loss of sensation in chronic itch conditions, due to desensitization. This evidence suggests that the search for new, putative mediators of itch must be continued in order to establish a greater understanding of how clinical pruritic conditions may be treated.

A general overview of many of the possible mediators of itch, as previously described in the literature, was given in Chapter 1.3. The studies presented in this chapter were focused on a few of the potential receptor targets that may be associated with evoking itch. Many more potential receptor targets have been, and will be in the future, identified, and the current study must be seen as a work in progress, in which there are many more possibilities to investigate.

5-HT

5-HT was identified as a compound capable of inducing itch in humans (Fjellner & Hagermark, 1979). However, the sensation generated was described as being less intense than that evoked by histamine. For this reason, studies into the itch-inducing properties of 5-HT were relatively ignored until the 1990's. 5-HT-induced itch is of a

greater intensity when the amine is co-injected with PGE₂, as is also the case with histamine (Fjellner & Hagermark, 1979). Another similarity with histamine-induced itch is that the response is mast cell-dependent (Weisshaar *et al.*, 1999). However, 5-HT was taken more seriously in its own right as a mediator of itch following evidence showing that the 5-HT₃ receptor antagonist, ondansetron, is effective at relieving the itch associated with cholestasis (Schworer & Ramadori, 1993), and also the itch induced by spinally administered opioids during epidurals (Borgeat & Stirnemann, 1999; Kyriakides *et al.*, 1999). 5-HT₃ receptor antagonists were also shown to reduce the itch induced by experimentally administered 5-HT (Weisshaar *et al.*, 1997).

Unlike histamine, 5-HT is not stored in human mast cells, but is released from platelets during platelet aggregation. However, rodent mast cells do contain 5-HT (Hagermark, 1992). Intradermal administration of 5-HT was shown to evoke scratching in mice (Andoh & Kuraishi, 2000; Inagaki *et al.*, 2001) and rats (Thomsen *et al.*, 2001). Studies so far suggest that 5-HT induced scratching in mice is 5-HT₂ and not 5-HT₃ receptor mediated, in contrast with the situation in man (Nojima & Carstens, 2003; Yamaguchi *et al.*, 1999).

Substance P

Substance P is a neuropeptide synthesized and released from afferent nerves, which acts as a neuromodulator on mast cells (Hokfelt *et al.*, 1980). Administration of substance P into the skin of healthy human subjects elicits the sensation of itch (Fjellner & Hagermark, 1981; Hagermark *et al.*, 1978), which acts through the release of histamine from mast cells (Ebertz *et al.*, 1987; Jorizzo *et al.*, 1983). In studies on mice, substance P has also been shown to induce dose related scratching (Kuraishi *et al.*, 1995). However, it has been proposed that unlike in man, this action is not mast cell mediated but is possibly dependent on NK1 receptors located on the peripheral nerves (Andoh & Kuraishi, 1998). To date, no clinical evidence exists to support the suggestion that NK1 receptors are associated with the sensation of itch.

PAR-2

The first work using proteases as pruritogens was performed using spicules of the plant cowhage, *Mucuna pruriens*, inserted into the dermis of man. It was proposed that the active ingredient was the enzyme, mucunain. This enzyme is denatured and the itch inducing properties lost, after boiling the spicules (Shelley & Arthur, 1955).

The mechanism whereby proteases induce itch is still not clear. Trypsin and chymase elicit itch in man that is alleviated by histamine H₁ receptor antagonists and is presumed to be mast cell dependent (Hagermark *et al.*, 1972). Two other endogenous proteases, papain and kallikrein, evoke itch that is not effectively reduced by H₁ receptor antagonists (Hagermark, 1974).

Recent evidence provides a new possibility for the mechanism of action of pruritic proteases, as they have been shown to act directly on receptors, known as protease activated receptors (PARs). There are currently four recognised receptors, PAR-1, PAR-2, PAR-3 and PAR-4 (Macfarlane *et al.*, 2001; Vergnolle *et al.*, 2001a). These receptors have so far been associated with inflammatory responses; however, the PAR-2 has also been implicated in mediating the sensation of itch (Steinhoff *et al.*, 2003). The receptor is located on peripheral C-fibre nerves and is activated by trypsin released from mast cells (Steinhoff *et al.*, 2000). PAR-2 may present a novel mechanism by which the sensation of itch is detected by cutaneous nerves.

Compound 48/80

Compound 48/80 is a compound that causes mast cell degranulation and subsequent itch in man (Fjellner & Hagermark, 1981) and mice (Kuraishi *et al.*, 1995). The itch is a result of histamine release and is blocked by histamine H₁ receptor antagonists (Sugimoto *et al.*, 1998). Compound 48/80 is a useful experimental tool used to determine whether a proposed itch-inducing protocol involves the mast cell and subsequent release of histamine pathway, or is histamine independent. By administering compound 48/80 to degranulate mast cells, any itching induced by drugs is assumed to be independent of mast cells or histamine (Andoh *et al.*, 1998; Rukwied *et al.*, 2000).

The experiments in this chapter were designed to confirm the scratch evoking properties of putative itch inducing drugs in mice, and to attempt to elucidate novel mechanisms of action. The studies were also designed to establish whether the scratch-inducing protocols, developed for use in the histamine-mediated model, described in chapters 3 and 5, are also suitable for studying other pruritogenic drugs.

6.2 Materials and Methods

The procedure for inducing itch and measurement of scratching as used in this chapter is as previously described in chapter 2.1, unless otherwise stated. Experiments in this chapter were conducted in 124 female BalbC mice, 10 female ICR mice and 12 female C57/Bl6 mice.

6.3 Results

5-HT induced scratching in BalbC mice

5-HT evoked scratching that was dose-dependent in all six female BalbC mice studied when administered intradermally, as summarised in figure 6.1A. Scratching was not observed at doses below 3nmol, but was consistently observed with higher doses, reaching a maximum at 80nmol 5-HT. Above this dose there was no further increase, and often a decrease, in scratching. 5-HT-induced scratching reached a maximum response of 113 ± 13 bouts (mean \pm sem), with a mean apparent ED₅₀ of 11nmol.

Strain differences to 5-HT induced scratching

5-HT also evoked dose-dependent scratching in both female C57/Bl6 and ICR strains of mice. 5-HT evoked scratching in all six C57/Bl6 mice, with a mean apparent ED₅₀ to 5-HT of 4nmol (see figure 6.1B). The maximum scratching response evoked was 142 ± 30 bouts (mean \pm sem).

5-HT evoked scratching in all ten female ICR mice, with a mean apparent ED_{50} to 5-HT of 8nmol (see figure 6.1C). Maximum scratching response evoked was 93 ± 10 bouts (mean \pm sem).

The three strains of mice showed no significant difference in mean apparent ED_{50} to 5-HT ($P > 0.05$, Kruskal-Wallis test with Dunn's multiple comparison post-test). The mean maximal scratching responses evoked by 5-HT also showed no statistically significant difference between strains ($P > 0.05$, Kruskal-Wallis test with Dunn's multiple comparison post test), however, the trend suggests that the C57/Bl6 mice scratch to a greater degree than either of the other two strains in response to 5-HT.

Mechanism of 5-HT induced scratching in BalbC mice

5-HT₂ receptor subtype

5-HT induced (8nmol, i.d.) scratching was significantly reduced 30 minutes following cinanserin hydrochloride (5-HT₂ receptor antagonist, 5 mg kg^{-1} , i.p. $P < 0.05$, repeated measures ANOVA, $n=4$, figure 6.2A). 5-HT induced scratching returned to pre-treatment levels 90 minutes following antagonist. The same procedure repeated in the same mice, one hour later, provided the same result, with a reduction in scratching after 30, but not 90 minutes post-antagonist ($P < 0.05$, repeated measures ANOVA).

5-HT induced (8nmol, i.d.) scratching was not reduced in mice treated with vehicle for the antagonist (distilled water, i.p. $P > 0.05$, repeated measures ANOVA, $n=4$, see figure 6.2B).

5-HT₃ receptor subtype

5-HT induced (8nmol, i.d.) scratching was not significantly reduced 30 minutes following 3,tropanylindole 3,carboxylate methiodide (a 5-HT₃ receptor antagonist, 5 mg kg^{-1} , i.p.). The same procedure repeated on two more occasions did not reduce 5-HT evoked scratching responses ($P > 0.05$, repeated measures ANOVA, $n=4$, see figure 6.3A). The vehicle used for the antagonist did not reduce 5-HT induced scratching ($P > 0.05$, repeated measures ANOVA, $n=4$, see figure 6.3B).

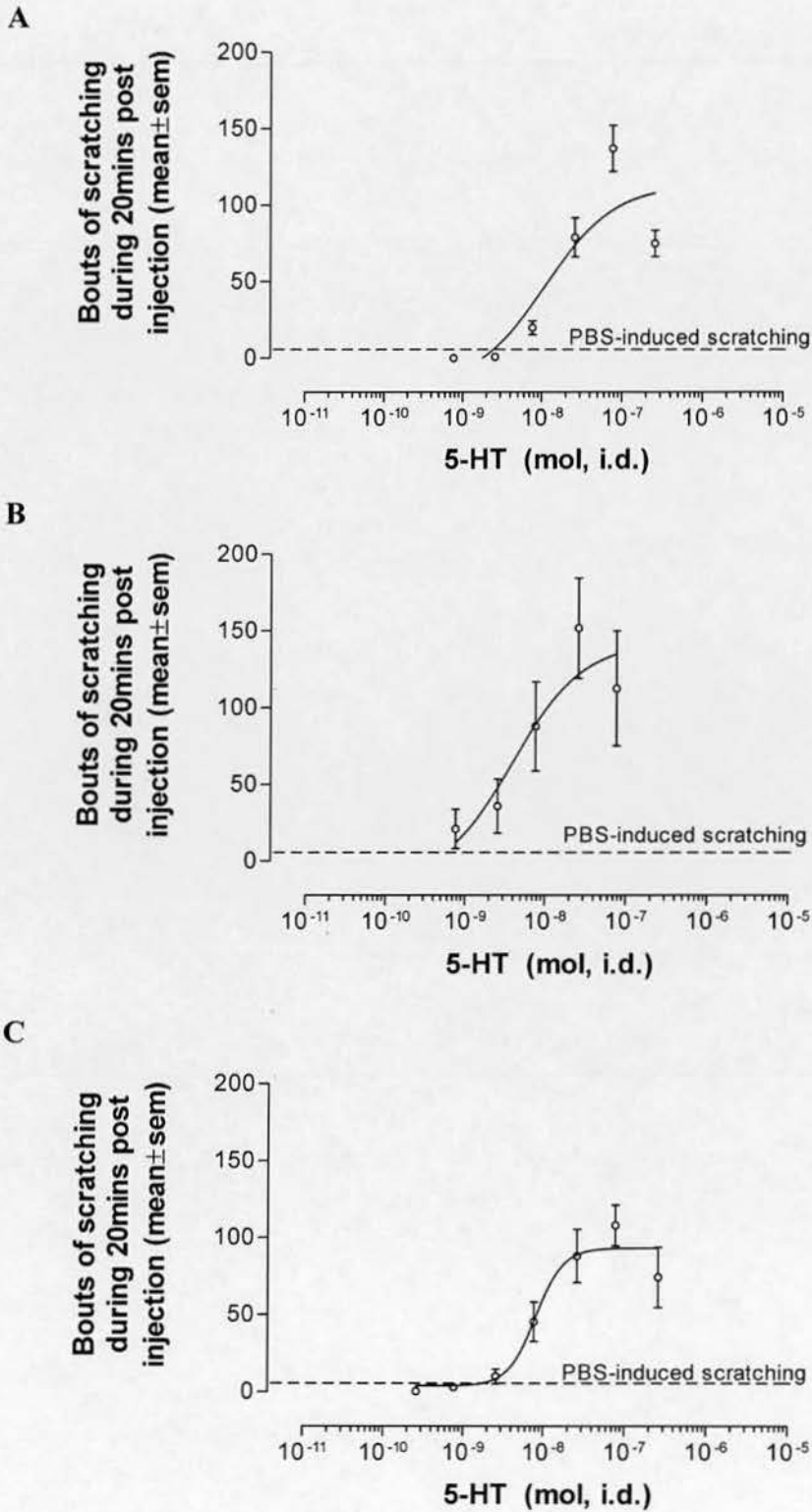


Figure 6.1 5-HT induced dose-dependent scratching in female mice: A: BalbC strain (n=6); B: C57/Bl6 strain (n=6) and C: ICR strain (n=10).

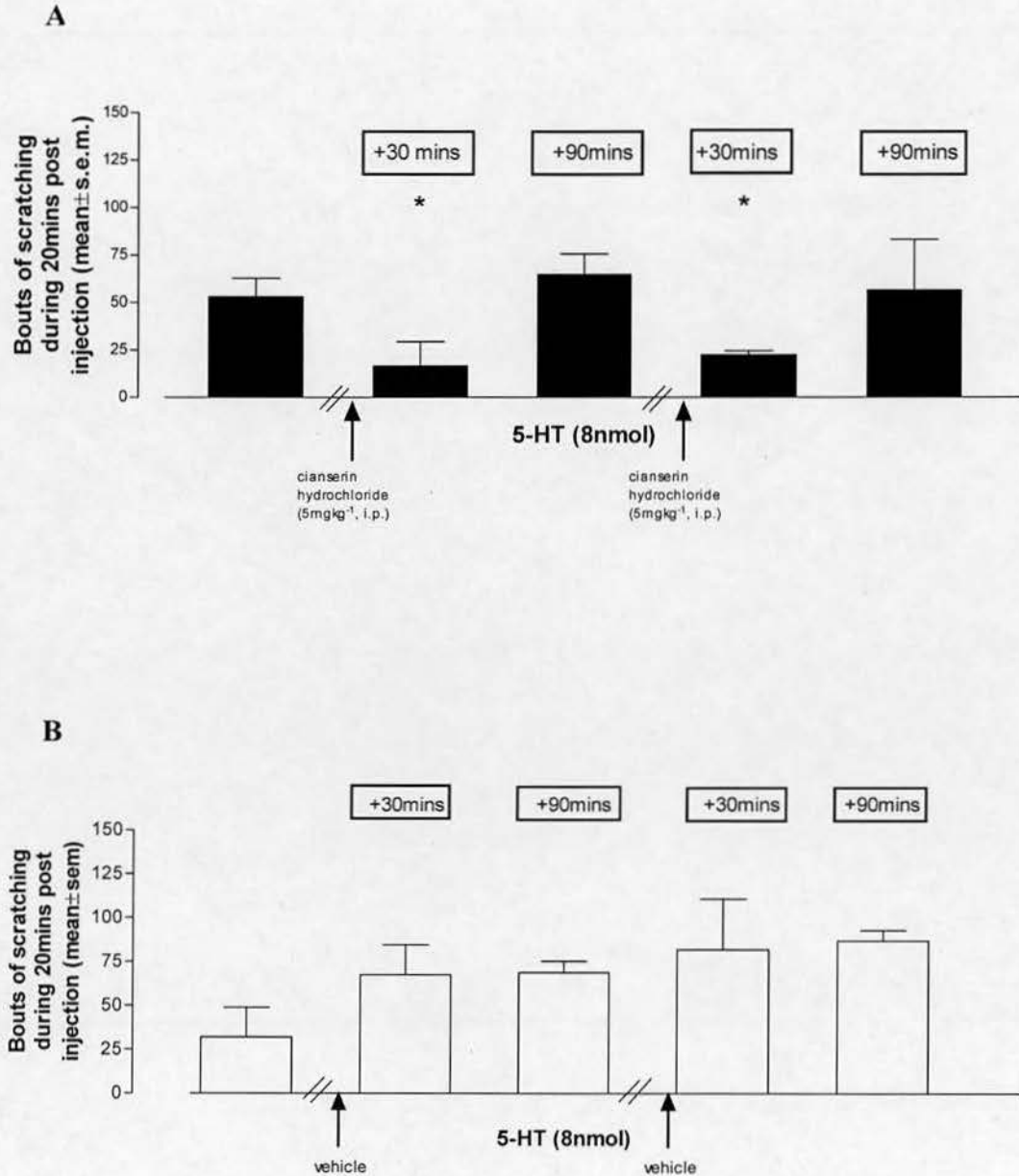


Figure 6.2 A: Pre-treatment with Cinanserin hydrochloride (5HT_2 antagonist, 5mgkg^{-1} , i.p.) significantly reduced 5-HT (8nmol , i.d., 30mins post antagonist) induced scratching compared with pre-treatment scratching response (* $P < 0.05$, repeated measures ANOVA, $n = 4$). 90mins post antagonist responses returned to pre-treatment levels. A second dose of antagonist gave the same result when repeated in the same group of mice. B: 5HT induced scratching was not significantly reduced in mice receiving vehicle ($P > 0.05$, repeated measures ANOVA, $n = 4$).

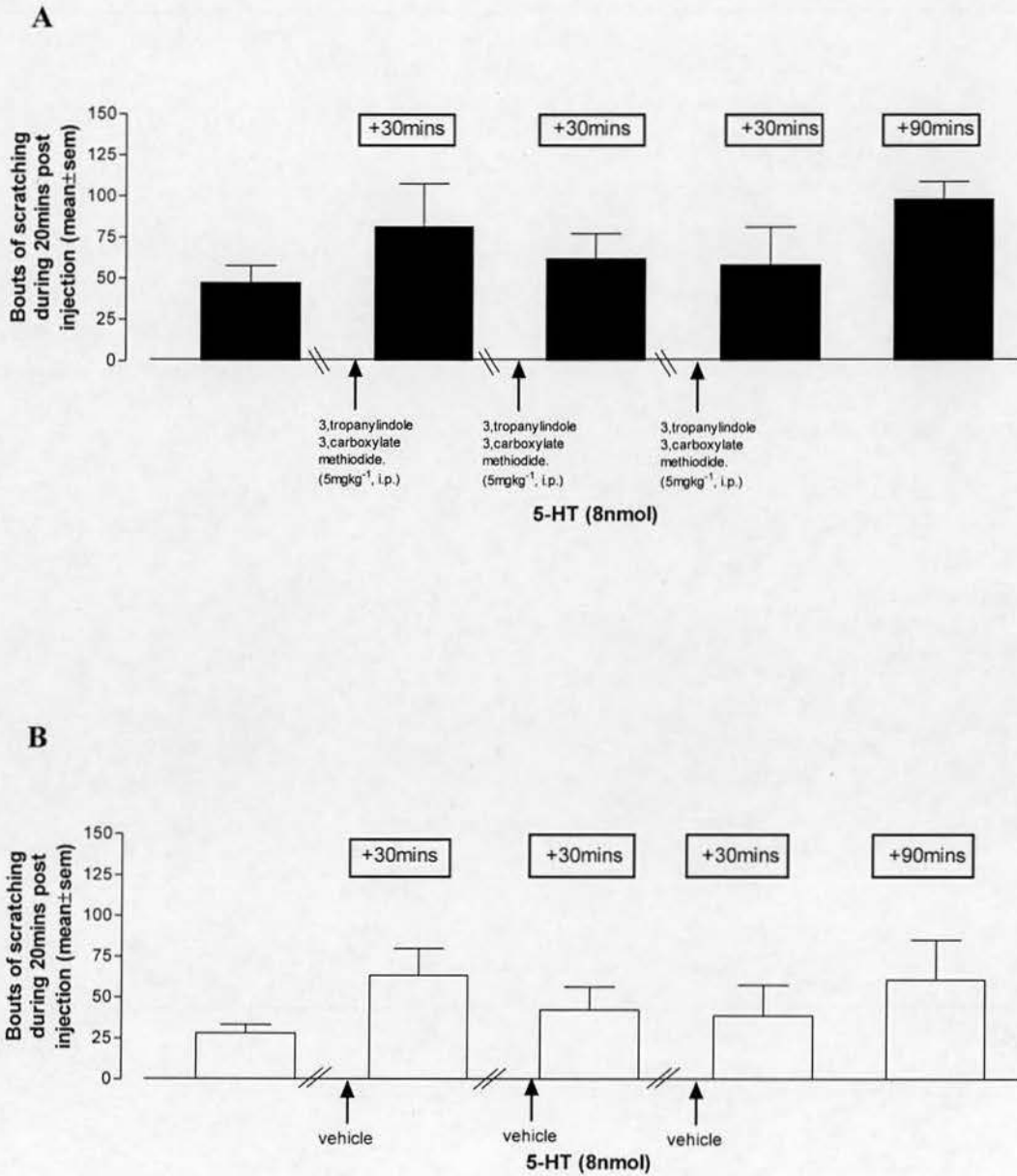


Figure 6.3 A: Pre-treatment with 3, tropanylindole 3, carboxylate methiodide (5HT₃ antagonist, 5mgkg⁻¹, i.p) did not significantly reduced 5-HT (8nmol, i.d.) induced scratching compared with pre-treatment scratching response ($P>0.05$, repeated measures ANOVA, $n=4$), after either of 3 separate antagonist injections. B: 5HT induced scratching was also not significantly reduced in mice receiving vehicle for the antagonist ($P>0.05$, repeated measures ANOVA, $n=4$).

Substance P induced scratching

Substance P evoked scratching at all doses tested (0.7-70nmol) in female BalbC mice that was significantly greater than that induced by vehicle (PBS, $P < 0.05$, Mann Whitney test, $n=4$, see figure 6.4A). The scratching was not dose-dependent following the doses used (maximum dose used was dependent on the solubility of the compound). The maximum scratching observed was 40 ± 18 bouts (mean \pm sem, 20nmol, median=26.5) following Substance P.

Substance P also evoked scratching, significantly greater than that induced by PBS, over the same dose range in female C57/Bl6 mice ($P < 0.05$, Mann Whitney test, $n=6$, see figure 6.4B). The trend in C57/Bl6 suggests that the response may be dose related, but the evidence from these experiments was inconclusive. The maximum scratching response in C57/Bl6 mice was 51 ± 24 bouts (mean \pm sem, 70nmol, median=32.5), indicating no significant difference from the maximal scratching response observed in BalbC mice ($P > 0.05$, Mann Whitney test).

Role of PAR-2 agonists in the induction of scratching in BalbC mice

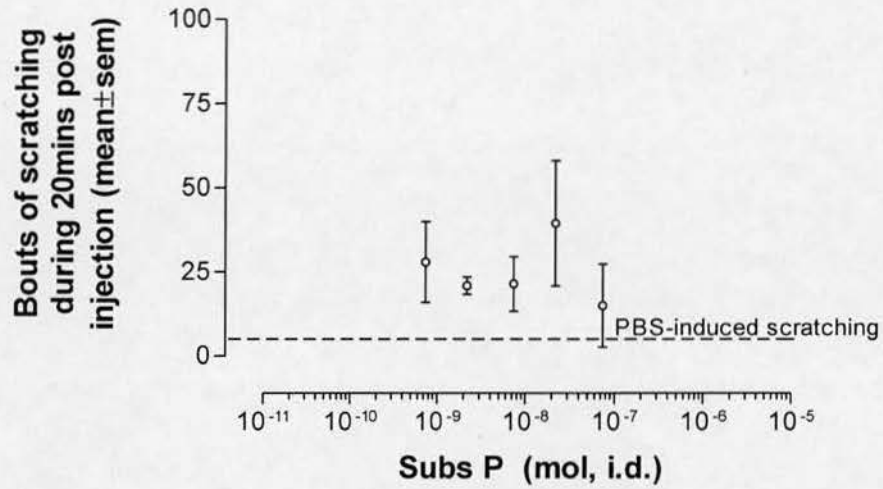
Trypsin-induced scratching

Trypsin (10 μ g-10mg, i.d.) induced dose-dependent scratching, in all six female BalbC mice studied, as shown in figure 6.5. Trypsin-induced scratching reached a maximum of 75 ± 6 bouts (mean \pm sem, $n=6$) with a mean apparent ED_{50} of 3.3mg. The mean maximum scratching response to trypsin was not significantly different to that observed for histamine as previously described in Chapter 3.3 (73 ± 12 bouts, $n=6$, $P > 0.05$, Mann Whitney test).

Trypsin (3mg, i.d.) induced scratching was not significantly lower in mice pre-treated with the histamine H_1 receptor antagonist, mepyramine (63 ± 13 bouts, 20mgkg⁻¹, i.p., median=60), versus vehicle treated mice (54 ± 21 bouts, median=42, $P > 0.05$, Mann Whitney test, $n=4$ in each group).

Trypsin (3mg, i.d.) induced scratching was also not significantly different in mice pre-treated with the histamine $H_{3/4}$ receptor antagonist, thioperamide (92 ± 20 bouts, 20mgkg⁻¹, i.p., median=82.5), versus vehicle treated mice (54 ± 21 bouts, median=42, $P > 0.05$, Mann Whitney test, $n=4$ in each group).

A



B

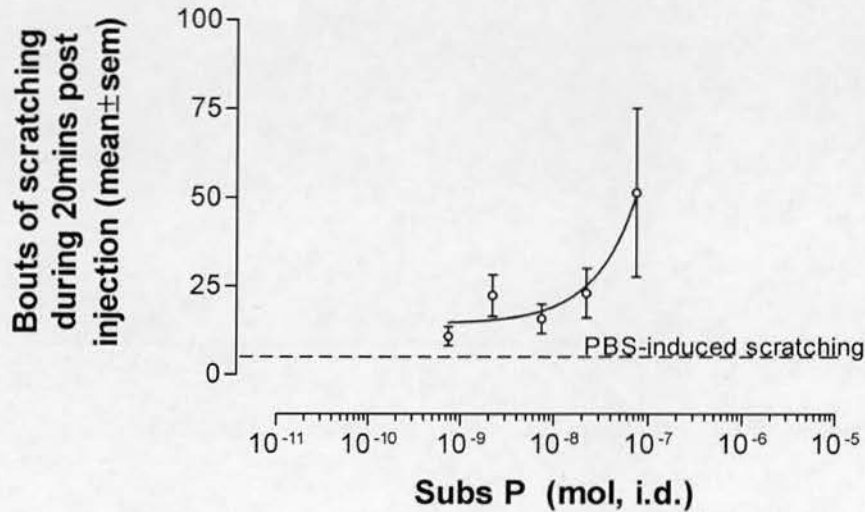


Figure 6.4 Scratching induced by Substance P in female mice: A: BalbC strain (n=4) and B: C57/Bl6 strain (n=6). In both strains substance P induced scratching that was significantly greater than that evoked by PBS (dashed line, $P < 0.05$, Mann Whitney test). Scratching was not dose dependent.

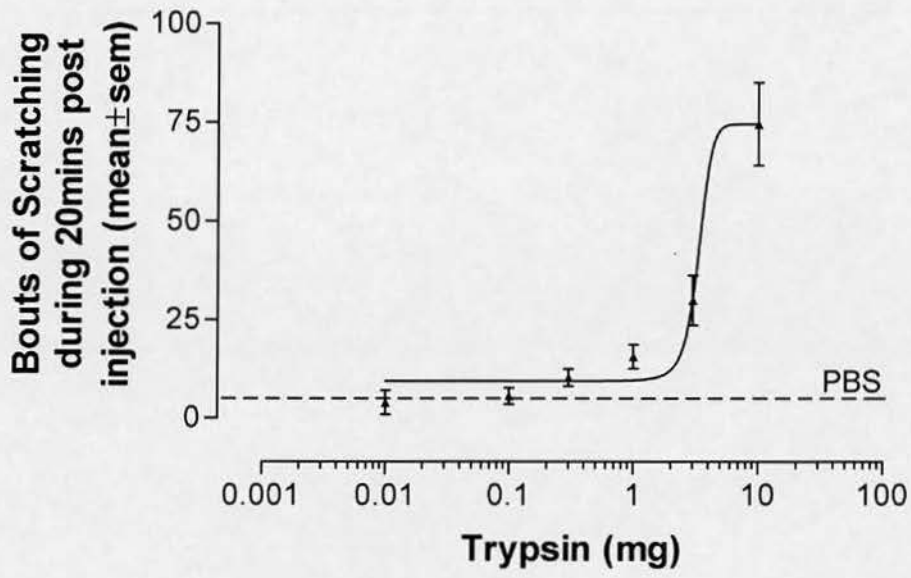


Figure 6.5 Scratching induced by trypsin in female BalbC mice (n=6). Scratching responses were dose dependent.

Papain-induced scratching

Papain (30 μ g-3mg, i.d.) induced dose dependent scratching, in all four female BalbC mice studied. Papain-induced scratching reached a maximum of 85 \pm 15 bouts (mean \pm sem, n=4) with a mean apparent ED₅₀ of 1.9mg. The maximum scratching in response to papain was not significantly different from that observed for histamine, as previously described in Chapter 3.3 (73 \pm 12 bouts, n=6, P>0.05, Mann Whitney test), as was the case for trypsin, described above.

Papain (0.3mg, i.d.) induced scratching was not significantly different in mice pre-treated with the histamine H₁ receptor antagonist, mepyramine (40 \pm 7 bouts, median=41, 20mgkg⁻¹, i.p.), in comparison with vehicle treated mice (55 \pm 8 bouts, median=58.5, P>0.05, Mann Whitney test, n=4 in each group, figure 6.6A).

Thioperamide (20mgkg⁻¹, i.p. histamine H_{3/4} receptor antagonist) pre-treated mice had a significantly reduced scratching response to papain (0.3mg, i.d.) compared to vehicle treated mice, 13 \pm 4 versus 52 \pm 6 bouts, median=13 versus 52 (mean \pm sem, P<0.05, Mann Whitney test, n=6 in each group, figure 6.6B).

SLIGRL-induced scratching

SLIGRL (PAR-2 agonist, 0.01 μ g-3 μ g, i.d.) induced dose dependent scratching in all four female BalbC mice studied. SLIGRL-induced scratching reached a maximum of 31 \pm 3 bouts (mean \pm sem, n=4) with a mean apparent ED₅₀ of 0.09 μ g. The mean maximum scratching response to SLIGRL was significantly less than that observed for histamine, as previously described in Chapter 3.3 (73 \pm 12 bouts, n=6, P<0.05, Mann Whitney test).

Mepyramine (20mgkg⁻¹, i.p.) pre-treated mice showed a significantly lower scratching response to SLIGRL (1 μ g, i.d.) in comparison with vehicle treated mice, 15 \pm 5 versus 37 \pm 4 bouts, medians=10 versus 33.5 (mean \pm sem, P<0.05, Mann Whitney test, n=8 in each group, figure 6.7A).

Thioperamide (20mgkg⁻¹, i.p.) pre-treated mice also had a significantly reduced scratching response to SLIGRL (1 μ g, i.d.) compared to vehicle treated mice, 3 \pm 1 versus 36 \pm 8 bouts, medians=2 versus 29.5(mean \pm sem, P<0.05, Mann Whitney test, n=4 in each group, figure 6.7B).

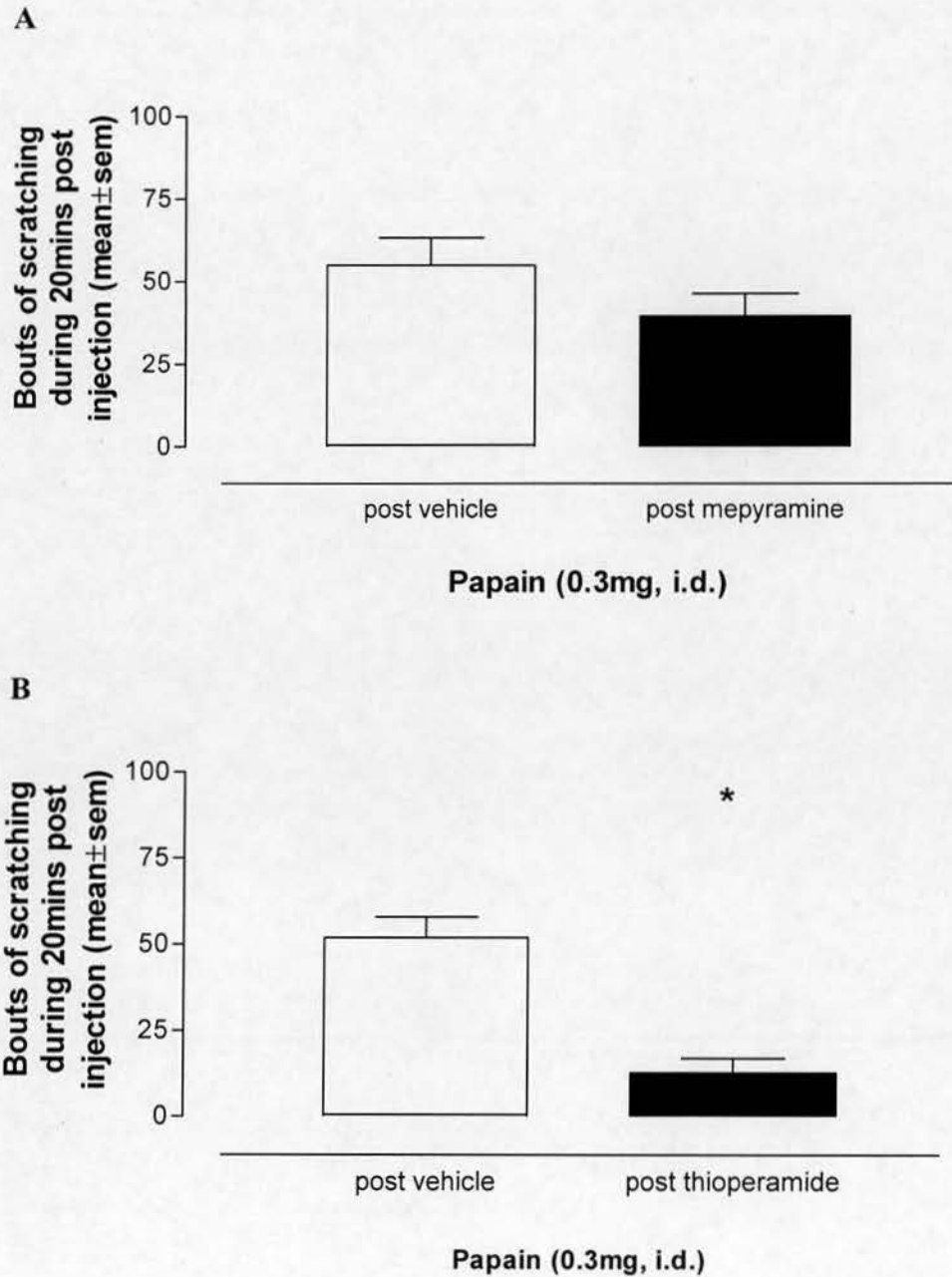


Figure 6.6 A: Pre-treatment with mepyramine (20mgkg^{-1} , i.p.) did not significantly reduce papain (0.3mg , i.d.) induced scratching compared with vehicle pre-treated mice ($P > 0.05$, Mann Whitney test, $n = 4$ in each group). B: Thioperamide (20mgkg^{-1} , i.p.) pre-treatment significantly reduced papain (0.3mg , i.d.) induced scratching compared with vehicle pre-treated mice ($*P < 0.05$, Mann Whitney test, $n = 6$ in each group).

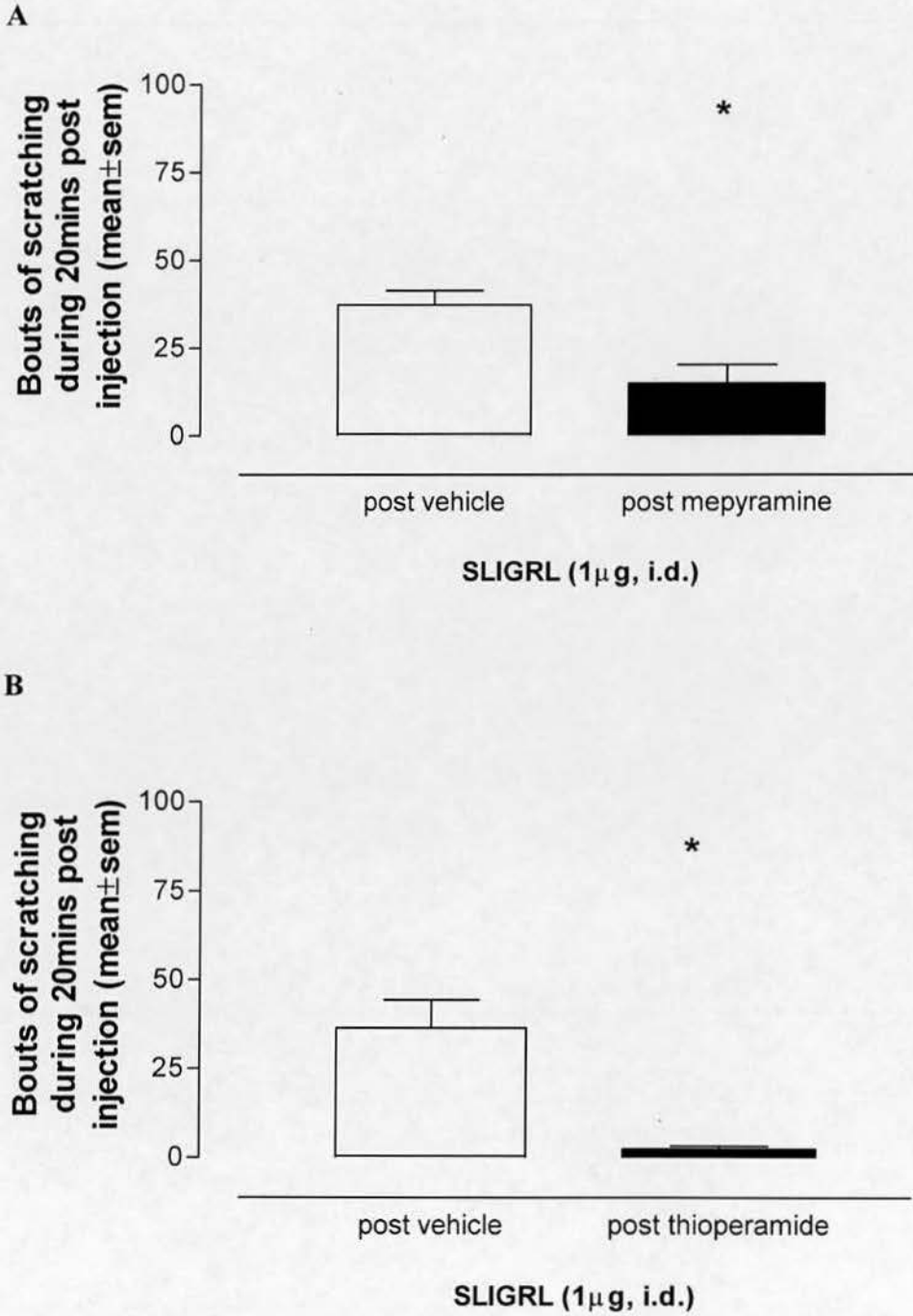


Figure 6.7 A: Mepyramine (20mgkg⁻¹, i.p.) pre-treatment significantly reduced SLIGRL (PAR-2 agonist, 1 μ g, i.d.) induced scratching compared with vehicle pre-treated mice (*P<0.05, Mann Whitney test, n=8 in each group). B: Thioperamide (20mgkg⁻¹, i.p.) pre-treatment significantly reduced SLIGRL (1 μ g, i.d.) induced scratching compared with vehicle pre-treated mice (*P<0.05, Mann Whitney test, n=4 in each group).

Compound 48/80 induced scratching in BalbC mice

Compound 48/80 evoked scratching that was dose-dependent in all four female BalbC mice studied, when administered intradermally, as summarised in figure 6.8. Scratching in response to compound 48/80 reached a maximum of 180 ± 16 bouts (mean \pm sem, $n=4$), with a mean apparent ED_{50} of $11 \mu\text{g}$. The maximum scratching response to compound 48/80 was significantly greater than that evoked by histamine (73 ± 12 bouts, $P < 0.05$, Mann Whitney test).

A repeat dose of compound 48/80 ($300 \mu\text{g}$, i.d.), one hour after the initial dose, significantly reduced the level of scratching observed, from 180 ± 16 to 27 ± 12 ($P < 0.05$, Mann Whitney test, $n=4$, medians= 180.5 and 22.5 respectively). This suggests that $300 \mu\text{g}$ of compound 48/80 is sufficient to cause a loss a scratching response, probably as a consequence of mast cell degranulation.

Compound 48/80 ($300 \mu\text{g}$, i.d.) pre-treatment caused a significant reduction in SLIGRL ($1 \mu\text{g}$, i.d.) induced scratching when compared to vehicle treated mice, as shown in figure 6.9. Pre-treatment with compound 48/80 ($300 \mu\text{g}$, i.d.) also caused a significant reduction in histamine ($3 \mu\text{mol}$, i.d.) induced scratching when compared to vehicle treated mice, 13 ± 3 versus 47 ± 14 bouts, medians= 12 versus 38 ($P < 0.05$, Mann Whitney test, $n=4$ in each group).

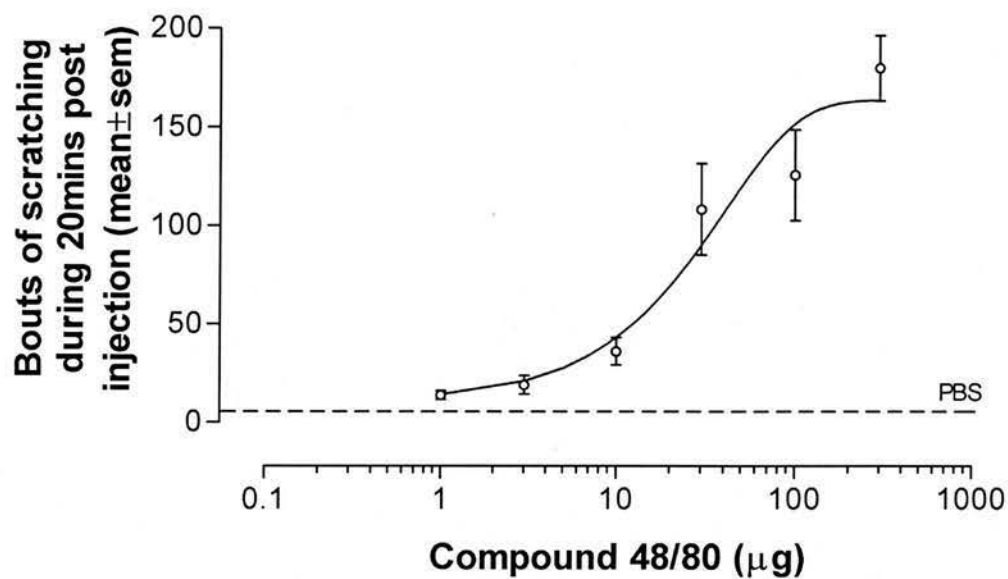


Figure 6.8 Compound 48/80 induced scratching in female BalbC mice (n=4). Scratching responses were dose dependent.

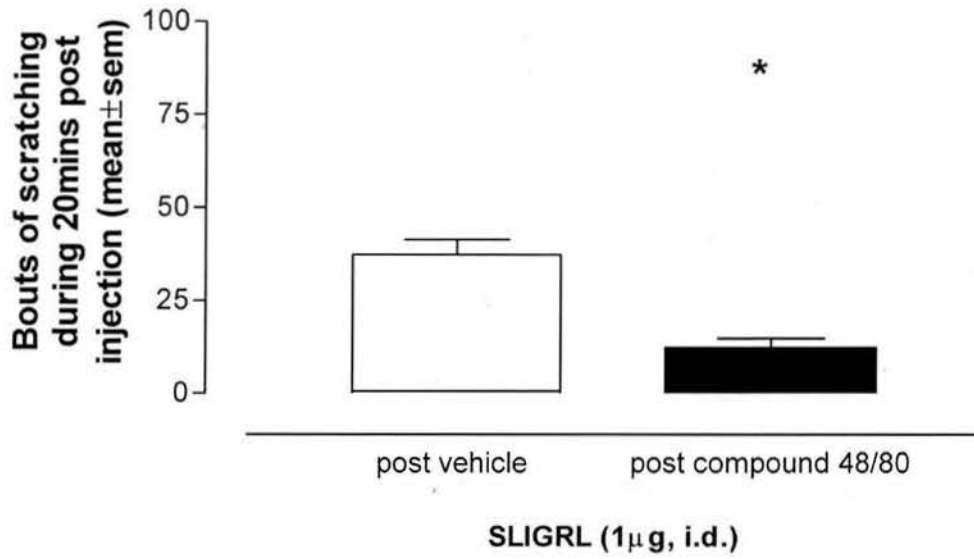


Figure 6.9 Pre-treatment with compound 48/80 (300µg, i.d.) significantly reduced SLIGRL (1µg, i.d.) induced scratching compared with vehicle pre-treated mice (* $P < 0.05$, Mann Whitney test, $n = 8$ in each group).

6.4 Discussion

The results from the studies in this chapter confirm that substances other than histamine cause scratching in mice. The evidence also suggests that the model of scratching developed in chapters 3 and 5 is suitable for use with pruritogenic drugs, in addition to histamine.

5-HT

The results showed that 5-HT induced dose-dependent scratching in female BalbC mice. The doses used in this study were similar to that used in previous studies both in man (Fjellner & Hagermark, 1979) and mice (Inagaki *et al.*, 2001). This suggests that 5-HT is a pruritogen in both species, with little species difference, unlike the differences in histamine response described in Chapter 3. The results also indicate that there is no strain dependent difference between 5-HT induced scratching. The difference in mean apparent ED₅₀s to 5-HT between the three strains used was 2-3 fold. This contrasts with the 30-fold difference in mean apparent ED₅₀s to histamine, which illustrated a difference in scratching between strains (as described in chapter 3). This evidence implies that the 5-HT induced mechanism of scratching is consistent between strains, something that may not necessarily be the case with respect to histamine-induced scratching.

Evidence for the involvement of 5-HT as a mediator of itch in clinical conditions came from the antipruritic effect of ondansetron, a 5-HT₃ receptor antagonist, in itch associated with cholestasis (Schworer & Ramadori, 1993) and also the itch induced by spinally administered opiates during epidural (Borgeat & Stirnemann, 1999; Kyriakides *et al.*, 1999). The results of this study do not support the findings from clinical human studies; there was no evidence of 5-HT₃ receptors being involved in 5-HT induced scratching. The results of these studies do, however, support the findings from previous murine studies that suggest a role for 5-HT₂ receptors in the scratching evoked by 5-HT (Nojima & Carstens, 2003; Yamaguchi *et al.*, 1999). Although these findings suggest a species difference between human and murine models of 5-HT induced scratching, much more work in the future will be required to

clarify whether any other 5-HT receptor subtypes are involved in the induction of scratching by 5-HT. The role of 5-HT in clinical itch may then become clearer.

Substance P

Substance P is the most widely investigated neuropeptide with respect to itch. The findings from the present study did not support the evidence previously presented in the literature. Dose-dependent scratching was not evoked in BalbC or C57/Bl6 mice at the doses used (maximum dose was dependent on the solubility of the drug). This contradicts the previous evidence in mice, in which substance P elicits dose related scratching responses, in doses similar to those used in this study (Kuraishi *et al.*, 1995). Although the present study failed to clearly elicit dose dependent scratching, the scratching that was evoked was of a level significantly greater than that induced by control. One possible explanation of the current findings would be that the itch is evoked via release of a secondary compound, most likely histamine from mast cells. This would support findings from human studies that substance P acts through mast cell degranulation (Ebertz *et al.*, 1987; Jorizzo *et al.*, 1983), and may explain an 'on or off' response, depending on whether the dose of substance P was sufficient to result in mast cell degranulation.

PAR-2

Protease-activated receptors are a group of 4 receptor subtypes that operate due to the cleaving effect of the protease on the N-terminus of the receptor protein, this action 'unteathers' the protein, which acts via a G-protein coupled mechanism (Vergnolle *et al.*, 2001b). One of these receptors, PAR-2, is known to be activated by trypsin, on peripheral nerve terminals (Steinhoff *et al.*, 2000). Trypsin itself has been shown to possess itch inducing properties (Hagermark *et al.*, 1972). In the present study, trypsin was shown to evoke dose dependent scratching in mice, consistent with the evidence in humans. The scratching evoked by trypsin was shown to be independent of mepyramine and thioperamide (histamine H₁ and H_{3/4} receptor antagonists respectively). Another itch inducing protease, papain, not active at the PAR-2 also evoked dose dependent scratching, again supporting the evidence from human studies (Hagermark, 1974). Scratching induced by papain was shown to be mediated,

at least in part, via histamine H_{3/4} receptors, but not an H₁ mechanism. This evidence suggests that trypsin induces scratching via a PAR-2 mechanism, distinct from scratching evoked by other proteases such as papain, which act through a histamine dependent mechanism.

The selective PAR-2 agonist, SLIGRL, induced dose-dependent scratching. However, scratching induced by SLIGRL was significantly reduced by both mepyramine and thioperamide, suggesting that PAR-2 operates via a histamine dependent pathway, most likely through mast cell activation. This evidence suggests that the scratching induced by trypsin, may be partly due to its action on PAR-2 receptors on mast cells, but also partly due to a non-histamine related mechanism, presumably through its proteolytic action on other surrounding anti-inflammatory cells.

Compound 48/80

The mast cell degranulator compound 48/80 induced dose dependent scratching in female BalbC mice, consistent with the evidence in the literature in both man (Fjellner & Hagermark, 1981) and mouse (Kuraishi *et al.*, 1995). As expected, a large dose of compound 48/80 resulted in a loss of further responses to the drug, as the mast cells had already been degranulated. This study also showed that subsequent injections of histamine and the PAR-2 agonist, SLIGRL, were significantly reduced. This evidence supports the earlier suggestion that SLIGRL, acts on PAR-2 on mast cells, and that prior degranulation results in a loss of response. SLIGRL would appear unable to act directly on sensory nerves via PAR-2 to elicit scratching.

Summary

The findings from the present studies show that scratching can be induced in mice by compounds, other than histamine known to be pruritogenic in humans. 5-HT, substance P, compound 48/80, trypsin, papain and the PAR-2 agonist, SLIGRL, were shown to cause scratching. 5-HT elicits a scratching response independent of histamine release, whereas the other compounds were histamine-dependent. PAR-2 activation results in scratching in mice, but the present results suggest this pathway is also histamine dependent, and probably involves mast cell degranulation. Future experiments on these and other compounds will almost certainly uncover new

mechanisms of itch induction, both dependent and independent of histamine release. This may result in the discovery of novel drug approaches to the treatment of clinical pruritic conditions.

Chapter 7

Chronically Induced Scratching in mice

7.1 Introduction

The experiments described in this thesis have so far focussed on the induction of acute scratching behaviour. Although such models are useful in determining the pruritic effect of various endogenous compounds they do not mimic clinical pruritic states in which the itch is of a considerably longer timescale than 15-20 minutes. Acute models of scratching do not lead to pathophysiological changes, such as those associated with chronic clinical itch. In order to investigate the role of putative antipruritic compounds it is necessary to develop chronic itchy conditions in animal models that are similar in pathophysiology to clinical states.

The ideal solution to chronic itchy conditions in mice is to find strains of mice in which these conditions exist naturally. So far only one such model exists: the NC/Nga mouse is a strain that spontaneously develops an atopic dermatitis-like condition, unless housed in a specific pathogen free environment, and shows increased, spontaneous scratching behaviour (Suto *et al.*, 1999; Vestergaard *et al.*, 2000). The development of gene technology may also provide the possibility in the future of creating transgenic mice with chronic itch disorders by over or under-expressing specific receptor subtypes or ligands. There is no evidence in the literature to date that such mice have been specifically developed.

Another approach to developing models of chronic itch is to evoke such skin responses using drugs. Evidence for long lasting pruritic actions of drugs administered to the skin is provided by the evidence that the contact sensitizer 2,4-dichloronitro benzene (DNCB) induces a scratching that persists for hours or days, rather than minutes (Laidlaw *et al.*, 2002). The purpose of the experiments in this study was to attempt to develop a model of chronic itching using DNCB in order to investigate the antipruritic actions of various compounds.

Compounds other than DNCB may also have the potential to cause chronic itch. One possible strategy that could evoke a chronic pruritic condition is the use of pro-inflammatories. One such pro-inflammatory is Freund's complete adjuvant (FCA),

heat killed micro-bacteria tuberculosis (Greenwald & Diamond, 1988), which is commonly used to evoke inflammation in joints to mimic arthritis (Gauldie *et al.*, 2001; Grubb *et al.*, 1991). If such a compound were administered to the skin, one can test the hypothesis that the subsequent inflammatory response may evoke an elevated scratching response.

A second potential target for evoking chronic itch is the PAR-2 receptor (Macfarlane *et al.*, 2001; Vergnolle *et al.*, 2001a). It has been shown that proteases, such as trypsin and papain, evoke acute scratching in mice, as does the PAR-2 receptor specific agonist SLIGRL (see chapter 6 for more details). One of the studies within this chapter investigates the hypothesis that repeated administration of papain or SLIGRL leads to the induction of a chronic pruritic condition.

The development of chronic itchy conditions provides a background upon which putative antipruritic drugs can be tested. In Chapter 5 of this thesis, the antipruritic effects of histamine H₁ and H₃/H₄ receptor specific antagonists were examined in detail. The development of a chronic itchy model would enable further investigation of the antipruritic effect of these drugs (mepyramine and thioperamide) to be studied.

Recently, a new antipruritic drug has been developed, tacrolimus. This compound is topically administered as a cream, which is effective in relieving the itch of atopic dermatitis in man (Cheer & Plosker, 2001) and also reduces scratching in NC/Nga mice (Hiroi *et al.*, 1998). Tacrolimus acts to stabilise T-cells, which are the early immune response cells. Tacrolimus is believed to bind to a protein within the T-cell and prevent up-regulation of genes of pro-inflammatory proteins (Pustisek *et al.*, 2002). The antipruritic effect of tacrolimus on drug induced chronic itch conditions will also be investigated in this chapter.

7.2 Materials & Methods

The protocols for the induction and recording of scratching using DNCB, and the administration of antipruritic drugs to DNCB-treated mice are described in chapter 2.2. Antagonists used in this chapter were mepyramine maleate (H₁ antagonist),

thioperamide maleate (H_3 and H_4 antagonist) and tacrolimus cream (1% and vehicle, Fujisawa, Osaka, Japan).

The chronic itch model based on the administration of FCA (1mgml^{-1} , heat killed *Mycobacterium tuberculosis* suspended in heavy paraffin oil) involved a protocol of 2 doses of FCA (0.1ml, intradermally) on days 0 and 3. The models based on papain (topically, 2.1 units ml^{-1}) and SLIGRL ($1\mu\text{g}$, i.d., in distilled water) involved application on a twice-daily basis, for the period between days 0-10.

Experiments in this chapter were conducted in a total of 72 female BalbC mice and were performed in the animal facility, 1 George Square.

7.3 Results

DNCB-induced chronic scratching in female BalbC mice

DNCB evoked a chronic skin response in all six female BalbC mice studied. DNCB (1% in ethanol) was administered topically to the area around the back of the neck on days 0 and 3. The skin response consisted of hair loss from the back of the neck as well as dryness and blistering of the skin. The hair loss and dryness of the skin was apparent from day 5 and lasted until approximately day 20, at which point the hair around the area began to re-grow. The dryness of the skin resulted in occasional minor bleeding of the skin, however, the mice appeared to be in no general state of distress. The only behavioural change observed in the mice was the induction of scratching (see figure 7.1A). Scratching behaviour was not observed prior to treatment with DNCB on day 0. By day 5 a noticeable, but not statistically significant level of scratching was observed (3 ± 1 bouts of scratching, mean \pm sem). The level of scratching increased steadily until day 10, reaching a peak of 7 ± 3 (median=5) bouts, on that day. Over the period between day 10 and day 17 the mean level of scratching remained consistently above 5 bouts, per 10-minute observation period. Beyond 17 days the mean level of scratching began to fall markedly. Due to the relatively small sample sizes and large degree of variability there was at no time

period a statistically significant level of scratching versus pre-treatment levels ($P>0.05$, one-way ANOVA, $n=6$). Despite this, the clear trend in scratching induced by DNCB indicated that this was a suitable scratch model from which to compare other chronic pruritogens and also investigate the effects of putative itch relieving compounds.

Ethanol, the vehicle for dissolving DNCB, did not induce the same pattern of scratching as DNCB in female BalbC mice (see figure 7.1B, $n=4$). There was no increase in scratching between days 5 and 10 in contrast to the scratching observed in DNCB treated mice, scratching in the ethanol treated group reached a peak of 3 ± 2 bouts, on day 15. There was also no hair loss or change in condition of the skin, unlike that observed in the DNCB treated group.

Effects of change in dose regimen on DNCB-induced scratching

A three-dose regimen of DNCB treatment was also studied, with topical application on days 0, 3 and 6 ($n=8$). The skin responses observed following the three-dose protocol was similar to that previously described following two doses; there was total hair loss from the treated area, associated with dryness and blistering of the skin, with occasional bleeding. The skin response was also observed between days 5 and 20, as described above. Scratching increased steadily up to day 9, reaching a peak level of 23 ± 3 bouts (mean \pm sem, median=20), significantly greater than the level of scratching induced by either the two dose regimen or ethanol (see figure 7.2A, $P<0.05$, Kruskal-Wallis test with Dunn's post test). Despite peaking at a higher level, scratching fell away and by day 12 had decreased to 6 ± 2 bouts. As was observed in the two-dose protocol, scratching remained consistently above 5 bouts up to day 17, after which scratching became more inconsistent.

A study into the effects of a four-dose regimen of DNCB application (on days 0, 3, 6 and 12) provided very similar results to that observed following the three-dose study. A characteristic loss of hair, and skin response was observed in all 8 mice studied. Scratching levels increased steadily up to day 10, peaking at 18 ± 3 (median=20) bouts, statistically significantly greater than that observed in the ethanol group (see figure 7.2B, $P<0.05$),

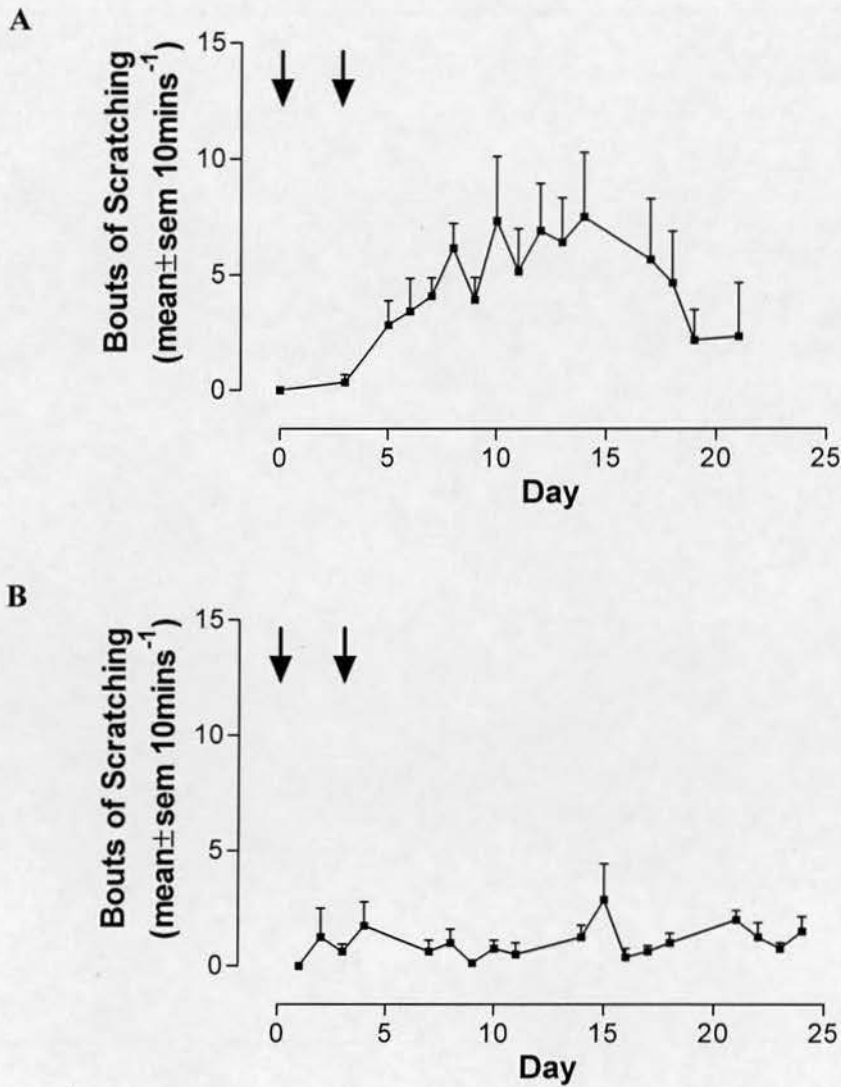


Figure 7.1 Chronic scratching induced by: A, DNCB (1%, topically, n=6, administered on days 0 and 3) and B, Ethanol (vehicle for DNCB, n=4). DNCB treated group increase scratching response up to day 10, followed by consistent plateau of response up to day 17. Vehicle treated mice do not increase scratching levels in the same pattern. Arrows indicate time of DNCB administration.

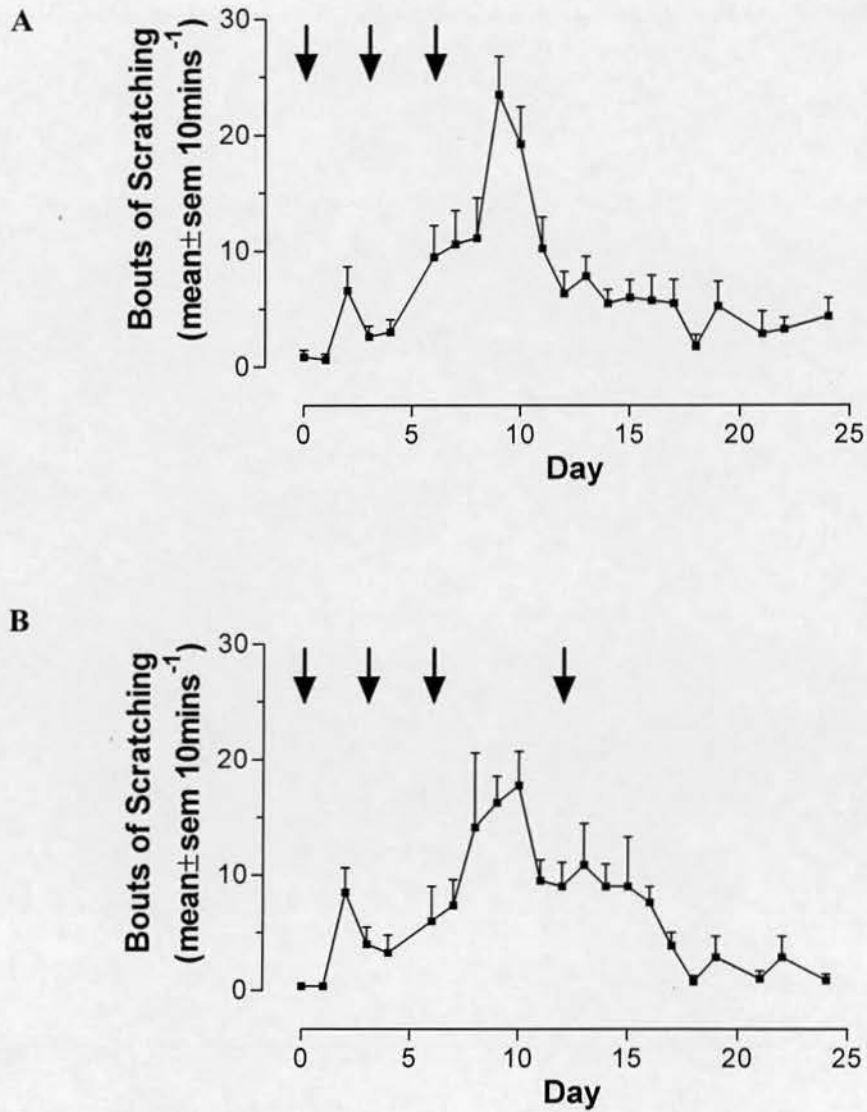


Figure 7.2 Chronic scratching induced by DNCB (1%, topically) using A; 3-dose regimen (days 0, 3 and 6, $n=8$) and B; 4-dose regimen (days 0, 3, 6 and 12, $n=8$). Both groups show a peak scratching response on day 10, with a subsequent plateau of scratching response between days 12 and 17. Arrows indicate time of DNCB administration.

but not significantly different from that induced by either the 2 or 3-treatment protocol ($P>0.05$, Kruskal-Wallis test with Dunn's post test). As was observed with the 2-dose group, scratching levels fell by day 11, to below 10 bouts. After this time, there was again a consistent scratching response over the following few days, until day 17 when scratching levels fell and became more variable.

Chronic scratching induced by PAR-2 receptor agonists

SLIGRL ($1\mu\text{g}$, i.d.) was administered daily to the back of the neck of six female BalbC mice. Scratching responses were observed and recorded on a twice-daily basis (see figure 7.3A). The treatment protocol took place over 11 days with mice being treated on days 0, 1, 2, 3, 7, 8, 9 and 10. After the treatment protocol ended mice were observed for scratching for the following 13 days. Directly following SLIGRL treatment scratching was observed, indicating SLIGRL to be mediator of itch (as previously described in Chapter 6). Following SLIGRL treatment scratching ranged from 6 ± 2 to 21 ± 4 bouts. The mean scratching response to SLIGRL over the 8 treatment days did not differ significantly ($P<0.05$, Kruskal-Wallis test with Dunn's post test), however, the trend from the data indicate that over the course of consecutive treatment days the mice scratch to a greater level following each administration; over days 0-3, scratching increased from 11 ± 2 to 16 ± 3 bouts and over the treatment days 7-10, scratching increased from 6 ± 2 to 17 ± 3 bouts. Scratching prior to treatment on each day was also observed, in order to determine if there was any chronic increase in the background level of scratching due to the SLIGRL administration. Background scratching peaked on day 3 at 4 ± 2 bouts, not significantly greater than that of the peak response to the ethanol control discussed previously ($P>0.05$, Mann-Whitney test). The mice from this study showed no behavioural changes other than those discussed and there were no apparent changes in the condition of the skin.

Papain (2.1 units ml^{-1} , topically) was applied daily to the back of the neck of six female BalbC mice. Scratching was observed and recorded on a twice-daily basis (see figure 7.3B). The treatment and observation protocol were as previously described for the study involving SLIGRL. Immediately after papain administration

scratching was consistently observed, indicating papain to be a mediator of itch (as previously described in Chapter 6). Scratching following papain treatment ranged from 7 ± 2 to 35 ± 4 bouts. The mean scratching response to papain over the eight treatment days did not differ significantly ($P<0.05$, Kruskal-Wallis test with Dunn's post test), however, the trend from the data indicate that over the course of consecutive treatment days the mice scratched to a greater level following each administration, as occurred with SLIGRL. Scratching prior to papain treatment on each day was also observed, in order to determine if there was any increase in the background level of scratching associated with papain administration. Background scratching peaked on day 10 at 9 ± 3 bouts, not significantly greater than that of the peak response to the ethanol control discussed previously ($P>0.05$, Mann-Whitney test). The background responses to papain, although not statistically significant, do appear to show a small elevation in scratching, however, this slight increase in scratching response (mean scratching remained above 5 bouts over the period day 8-11) diminished within a day or two of stopping the papain and did not provide the same plateau of scratching response as generated by DNCB treatment. The mice from this study showed no behavioural changes other than those discussed and there were no apparent changes in the condition of the skin.

Chronic scratching induced by Freund's Complete Adjuvant (FCA)

Freund's complete adjuvant (FCA, 0.05ml, i.d. on days 0 and 3) did not induce scratching in female BalbC mice, significantly greater than that induced by ethanol (see figure 7.4, $P>0.05$, Mann-Whitney test), scratching peaked at 4 ± 4 bouts on day 31. FCA caused no obvious changes to the skin, or any hair loss.

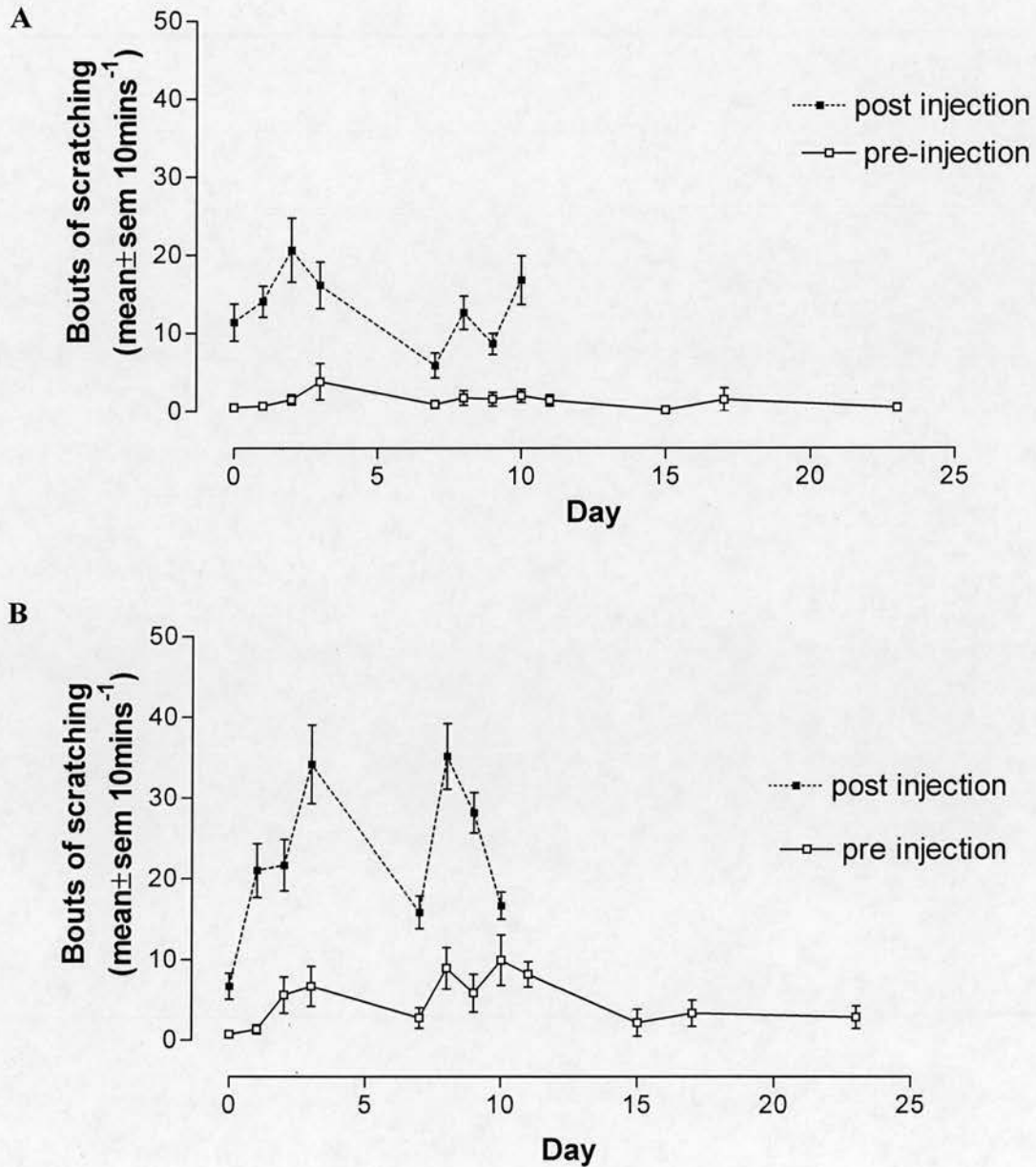


Figure 7.3 A; Chronic scratching induced by SLIGRL (PAR-2 receptor agonist, 1 μ g, i.d. administered on days 0, 1, 2, 3, 7, 8, 9 and 10, n=6). Scratching was induced following administration on each day. Background scratching (as measured prior to treatment) did not increase over the treatment period. B; Chronic scratching induced by papain (2.1 units ml⁻¹, topically, administered on days 0, 1, 2, 3, 7, 8, 9 and 10, n=6). Scratching was induced following administration on each day. Background scratching (as measured prior to treatment) did not increase over the treatment period.

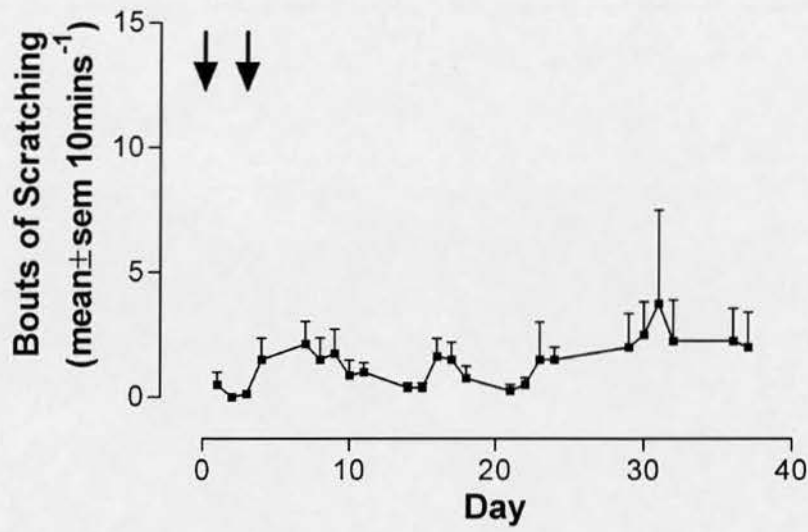


Figure 7.4 Chronic scratching induced by FCA (Freunds complete adjuvant, 1mgml⁻¹, i.d. n=4). Maximal FCA induced scratching was not significantly greater than that induced by ethanol. Arrows indicate time of FCA administration.

Effect of Tacrolimus on DNCB-induced scratching

Tacrolimus cream (1%, topically) was administered twice daily (days 6-11) to six DNCB treated mice. The background level of scratching (see figure 7.5A), measured by observing the level of scratching prior to tacrolimus administration on each day, increased steadily between days 3 to 12, reaching a peak of 15 ± 2 (median=15) bouts; not significantly different from the peak level from the two-dose DNCB treated group not receiving tacrolimus, described previously ($P > 0.05$, Mann-Whitney test). As well as scratching behaviour, there were also associated behavioural changes due to tacrolimus administration. The tacrolimus treated mice appeared to be hyperactive and uncomfortable, not only directly following treatment, but also before treatment on each day. This behaviour became more pronounced over the six treatment days, but began to subside within two days of ending the tacrolimus treatment. By the end of the study the behaviour was not different from mice that had not received tacrolimus. The trend from the data suggested that directly following tacrolimus administration, there was a reduction in the level of scratching (although at no time was this a significant reduction, $P > 0.05$, Kruskal-Wallis test with Dunn's post test). This reduction may, at least in part, be due to the associated change in behaviour mentioned previously. It appeared that the mice were uncomfortable and distressed; this may explain reluctance of the mice to scratch following treatment.

A separate group of DNCB pre-treated mice received the tacrolimus vehicle cream. In these mice there was no reduction in mean scratching directly following application of the cream, compared with background scratching levels (see figure 7.5B), in fact there was a slight increase on each day; this may be due to the presence of the cream on the back of the neck, and may simply be an attempt to remove the cream. The background scratching response followed a pattern similar to that observed in mice receiving no tacrolimus (or vehicle) cream, however, there is an indication from the data that the plateau of scratching response may be shortened.

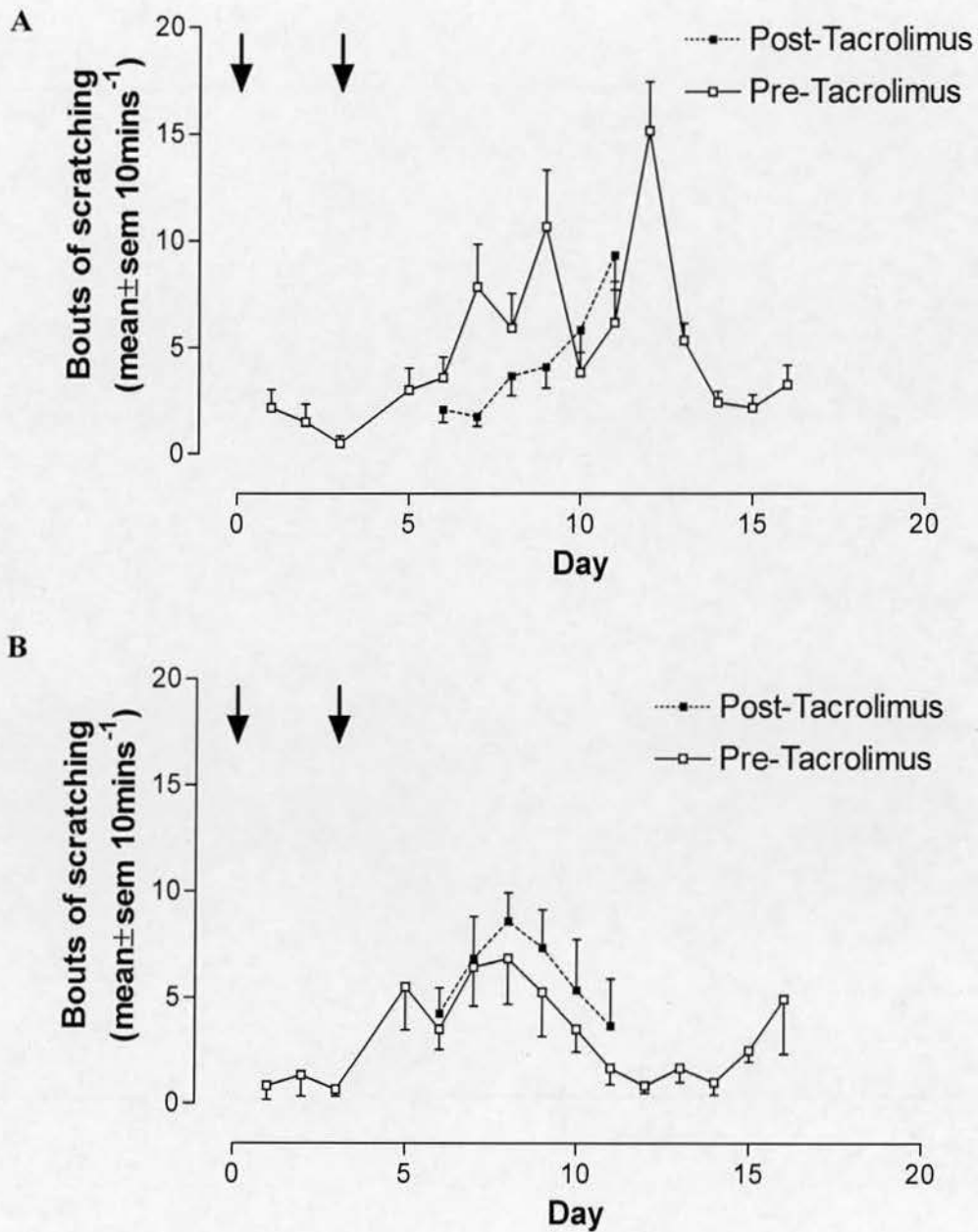


Figure 7.5 A; Tacrolimus (1%, topically, administered on days 6-11, $n=6$) did not reduce background scratching induced by DNCB (1%, topically, administered on days 0 and 3). Scratching directly following tacrolimus was reduced on the first four days of treatment but this effect was not seen following the last two days of treatment. B; Vehicle for tacrolimus (topically, administered on days 6-11, $n=6$) appeared to shorten the characteristic plateau of scratching response (usually lasting to day 17) induced by DNCB (1%, topically, administered on days 0 and 3). Scratching directly following vehicle was not reduced on any of the treatment days. Arrows indicate DNCB administration.

Effect of histamine receptor antagonists on DNCB-induced scratching

Mepyramine (H_1 receptor antagonist, 20mgkg^{-1} , i.p.) and thioperamide (H_3/H_4 receptor antagonist, 20mgkg^{-1} , i.p.) were administered twice daily on days 6-11 to two separate groups of mice that had been pre-treated with DNCB (on days 0 and 3). Background scratching responses to both drugs were not lower than those seen in mice receiving no antagonist. Scratching peaked at 13 ± 3 bouts (mean \pm sem) in the mepyramine group (see figure 7.6A) and 19 ± 5 bouts in the thioperamide group (see figure 7.6B). Both groups also showed the plateau of scratching response, which lasted for several days, as previously identified in the group receiving no antagonist. Although there was no reduction in background scratching, both groups of mice showed an apparent (but not statistically significant) reduction in scratching response directly following antagonist.

Combined antagonist treatment (20mgkg^{-1} mepyramine and 20mgkg^{-1} thioperamide, i.p.) eliminated scratching directly following administration (see figure 7.6C). Combined treatment also appeared to reduce background scratching, with a peak mean scratching response of 4 ± 2 bouts seen on day 14 (after the antagonist treatment had ended).

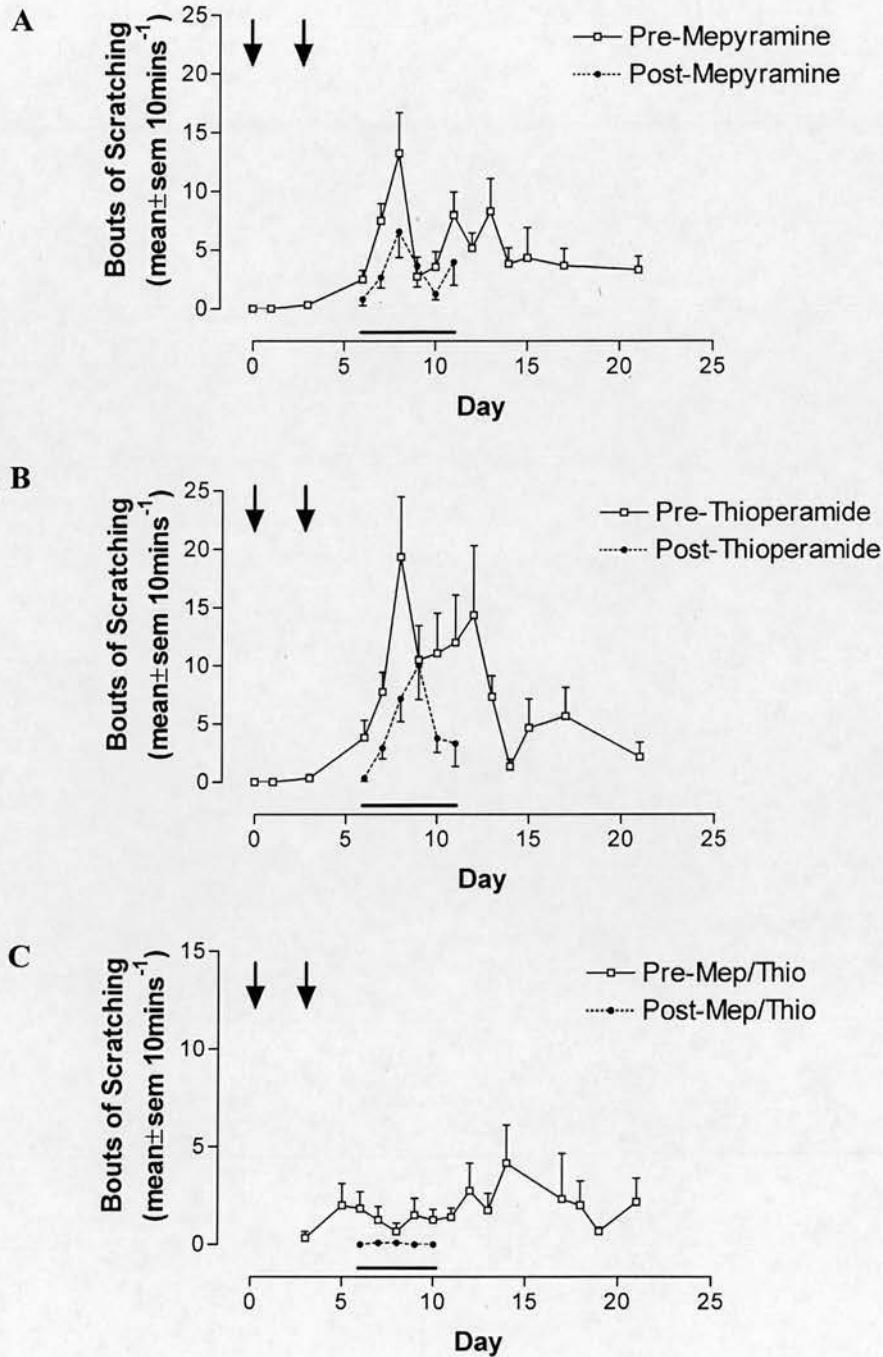


Figure 7.6 A; Mepyramine (H_1 antagonist 20mgkg^{-1} , i.d. administered on days 6-11, $n=6$) and B; thioperamide (H_3/H_4 antagonist 20mgkg^{-1} , i.d. administered on days 6-11, $n=6$) did not reduce background scratching induced by DNCB (1%, topically, administered on days 0 and 3), with the response still exhibiting the characteristic plateau of scratching. Scratching directly following each antagonist was reduced. C; Combined mepyramine and thioperamide ($n=6$) treatment eliminated scratching directly following antagonist and also eliminated the characteristic plateau of scratching response usually evoked by DNCB. Arrows indicate DNCB treatment. Solid line shows antagonist treatment.

7.4 Discussion

The evidence from the current study indicates that itch can be investigated in mice using chronically induced skin conditions, as well as the previously described acute models. This finding opens up new possibilities into the investigation of pruritus. Using acute models of experimentally induced scratching behaviour is of benefit in determining the pruritogenic properties of putative mediators of itch. However, such an approach is of more limited use when applied to specific pruritic conditions in man. For instance, there is no evidence in the literature to support the suggestion that any clinical chronic pruritic condition is due solely to increased dermal concentrations of any one mediator. Such chronic conditions are invariably due to changes in skin pathophysiology, and as such are likely to be the consequence of a range of endogenous mediators acting directly or indirectly on sensory nerves. Clinically, chronic pruritic conditions are therefore more difficult to understand and treat effectively. The development of chronic skin conditions, that cause itch, are therefore more likely to resemble the pathophysiology of certain chronic clinical pruritic conditions in man. The investigation of such models is of potentially greater benefit for the development of antipruritic compounds than are acutely induced models of scratching.

This study indicates that DNCB, applied topically, twice to the back of the neck of female BalbC mice, caused increased scratching over the course of approximately 17 days. The scratching provided a plateau of relatively consistent scratching over the period from day 10 to 17, over which time scratching remained consistently above a mean level of 5 bouts per ten-minute observation period. This scratching was also accompanied by a skin reaction, over the same timescale, consisting of hair loss, dryness and blistering of the skin. Treatment with ethanol (vehicle for DNCB), induced no skin response or behavioural changes. This evidence supports the previous literature implicating DNCB as a contact sensitizer in mice, inducing scratching over a period of hours and days, not minutes as is the case with the mediators discussed in previous chapters (Laidlaw *et al.*, 2002).

Due to variability in scratching obtained in different animals, no statistically significant evidence was generated to prove that DNCB induced increased scratching; for this reason it is important to identify the trends indicated from the study, whilst not over interpreting the data obtained and drawing premature conclusions. One major factor in the variability from this study is the amount of time for which each group of mice was observed. Each mouse was observed for only two ten-minute periods per day; this was in order to reduce the labour intensity of the process. Ideally, the mice would be observed over a longer, if not constant, time period. I suggest that due to the length of the study it is unreasonable to assume the mice scratched at a constant rate. It is likely that the mice had periods of reduced scratching throughout each day; possibly due to tiredness from excessive scratching or due to discomfort of the area, from either the skin condition itself or the increased scratching. To reduce the variability I think the most suitable approach would be to use the automated detector of scratching (see chapter 4). This would provide a constant recording of scratching and could reduce the variability generated from manually observing mice during 'inactive' periods of the day. Unfortunately, due to space restrictions within the animal facility such an approach was not possible during these studies.

Studies using a three and four dose regimen of DNCB also induced increased scratching responses. It was hoped that these increased doses would produce either a longer scratching response or that the plateau of scratching response would be higher, and therefore illustrate more clearly the pattern of response. The evidence from this study indicate that the three and four dose regimen evoke an elevated peak of scratching, around day 10, however this elevated peak diminishes within one or two days and from then on the pattern of response is very similar to that described for the two-dose protocol. As there was no clear elevation in the longer term level of scratching due to either the three or four dose regimen, further studies were conducted using the two-dose protocol as the standard chronic scratching state against which potential antipruritic drugs could be tested.

Attempts to develop a model of chronic scratching were also made using the protease enzyme, papain, the PAR-2 receptor agonist, SLIGRL and also the pro-inflammatory agent, FCA (Freunds complete adjuvant, heat killed *Mycobacterium tuberculosis*). Proteases have been implicated as mediators of scratching since the 1950's (Shelley & Arthur, 1955) and their actions are described in more detail in chapter 6. One possible receptor site at which proteases act to induce itch is at the PAR-2 receptor (Macfarlane *et al.*, 2001; Vergnolle *et al.*, 2001a). Both papain and SLIGRL have been shown to induce acute scratching behaviour (see chapter 6 for details) but results from the current study indicate that neither was capable of inducing a chronic itchy state equivalent to that evoked by DNCB. Both compounds continued to evoke scratching immediately following administration but neither drug produced an elevated long-term increase in scratching. As well as there being no increase in scratching there were also no other changes in skin condition (hair loss, scarification etc.)

FCA is a pro-inflammatory agent commonly used in this laboratory for the induction of experimentally induced arthritis in both rats and mice (Gauldie *et al.*, 2001; Grubb *et al.*, 1991). FCA was administered intradermally to assess whether a pro-inflammatory action would induce a chronic itch. From the results of this study there was no evidence to indicate that FCA was capable of evoking a scratch response in female BalbC mice.

The present studies focused on the use of drugs to induce chronic scratching in standard laboratory strains of mice. However, there is one strain, NC/Nga mice, that exhibit a spontaneous scratching response, which is considered to be similar in pathophysiology to atopic dermatitis (Suto *et al.*, 1999; Vestergaard *et al.*, 2000). Models of spontaneous scratching conditions as well as drug-induced models are both of potential use in the further investigation into antipruritic drugs. In the future it may be possible to develop a wider range of spontaneously itchy mice by using gene technology to over or under express receptor or ligand genes. This approach is potentially of greater experimental use because there would be a known cause of the itch as opposed to the situation with the NC/Nga mouse, where the exact

pathophysiology of the condition is not yet fully established. Spontaneous scratching in NC/Nga mice is reported to be alleviated by the antipruritic cream, tacrolimus (Hiroi *et al.*, 1998). Tacrolimus is also described as being effective at relieving atopic dermatitis in man, and is now commercially available as an antipruritic (Cheer & Plosker, 2001). The action of tacrolimus is based on its ability to act as a lymphocyte T-cell stabilizer. It is thought that tacrolimus binds to an intracellular protein within the T-cell and inhibits the promoter region of a range of early pro-inflammatory mediators, such as interleukins (Pustisek *et al.*, 2002).

In this study the antipruritic effect of tacrolimus was investigated (over the course of 6 days) in mice pre-treated with DNCB (2-treatment regimen). Tacrolimus (1%) did not reduce the background level of scratching compared with DNCB-induced scratching, described previously. Indeed, the scratching induced appeared to be greater in magnitude due to tacrolimus with the behaviour of the mice also changing; showing signs of anxiety, discomfort and hyperactivity. This behavioural response may be due to the dose of tacrolimus used as some reports suggest that the drug can induce an irritant effect (Pustisek *et al.*, 2002). From this study it is difficult to suggest whether there may have been any antipruritic effect of tacrolimus on mice treated with DNCB. The only evidence to support the antipruritic role of tacrolimus in this study comes from the first 4 days of tacrolimus administration, when a small but noticeable reduction in scratching could be seen, directly following tacrolimus treatment. Mice treated with tacrolimus vehicle showed no overt changes in behaviour following application of the cream. As we would expect from a vehicle drug, there were no changes in the level of scratching following administration of the cream. However, in the vehicle treated group there was an apparent reduction in the length of the plateau of scratching response compared to the pattern seen in DNCB treated mice receiving no antipruritic drug. This may indicate that the vehicle cream, although providing no immediate itch relief acts over the course of several days to reduce scratching, most probably due to moisturizing effects of the skin, reducing dryness and having a soothing effect. Further studies would be required using various concentrations of tacrolimus in order to determine whether the tacrolimus can reduce

the itch induced by DNCB, or whether DNCB induces its effects by a mechanism distinct from T-cell activation.

It has previously been shown that the histamine H₁ receptor antagonist, mepyramine, and the histamine H₃/H₄ receptor antagonist, thioperamide, are capable of significantly reducing histamine induced scratching (see chapter 5). The antipruritic effects of mepyramine, thioperamide and the combination were investigated in DNCB pre-treated mice. Mepyramine and thioperamide were both administered in the doses previously shown to reduce scratching (20mgkg⁻¹, see chapter 5). The results from the present study indicate that mepyramine and thioperamide (administered twice daily over days 6-11) reduce scratching immediately following their administration, however, they did not appear to reduce the background level of scratching, as measured by either peak scratching response or by duration of the plateau of scratching response, when compared to the control DNCB treated mice described previously. Interestingly, mice treated with the combination of mepyramine and thioperamide showed less background scratching during the treatment period; at no point did scratching reach a mean level of 5 bouts which was the level in control DNCB treated mice during the plateau phase. Scratching immediately following antagonist treatment was eliminated completely. This evidence suggests that a combination of histamine H₁ and H₃/H₄ receptor antagonists may provide an interesting new treatment of itch, both in animal models and also in human clinical conditions.

The development of models of chronic scratching, such as those described in this chapter, provides another new possibility for the investigation of putative antipruritic drugs, which may in time lead to the development of drugs of use clinically in man.

Chapter 8

Electrophysiological Studies of Pruritus

8.1 Introduction

The previous investigations described within this thesis have focused on the development of behavioural models of scratching to determine the sensation of itch. The problem of such an approach is that the evidence generated is subjective in two ways; firstly, the level of scratching evoked may be recorded differently over successive experiments (this variation is hopefully reduced by the development of the automated detector of scratching); secondly, we can not be sure that the same peripheral stimulus in two mice would elicit the same behavioural response. It is highly feasible that the central processing of the itch stimulus would evoke a variety of behavioural responses, dependent upon the individual being investigated, just as some people appear to be more sensitive to a given painful stimulus than others.

One technique, to reduce the variation generated by the central processing and subsequent behavioural response, is to attempt to record the activity of the peripheral afferent nerves innervating the murine skin, following an itch stimulus. The use of electrophysiological techniques allows the degree of response to be determined, independent of central processing. One electrophysiological study into itch in mice has previously been described in the literature. The study describes an increase in the number of action potentials, recorded from an exposed cutaneous nerve from the back of the neck, following intradermal administration of 5-HT (scratch evoking compound, as described in chapter 6 of this thesis) in two standard laboratory strains, ICR and ddY, of anaesthetised mice (Maekawa *et al.*, 2000). Moreover, the pattern of neural firing identified mirrors that seen in behavioural studies, with the increase in response starting after 3-4 minutes and lasting up to about 20 minutes. The two strains responded differently to another pruritogen, histamine. Histamine evoked a similar pattern of neural response to 5-HT in ICR mice; however, no increase in neural response was identified in ddY mice. This result may be explained by the strain related differences evoked by histamine in mice, as previously discussed in chapter 3 (Inagaki *et al.*, 1999). The authors proposed that the neural responses generated were, 'itch-associated responses of afferent nerves'. Further investigation

of itch-associated responses in electrophysiological studies would allow us to learn more about the pruritogenic effects of putative 'itch evoking' drugs.

Work in humans has been able to identify a population of 'itch specific C-fibres', located in human skin that respond selectively to histamine (Schmelz *et al.*, 1997). The suggestion that there is a population of nerves that respond specifically to pruritogenic stimuli is supported by work in cats showing a population of spinothalamic tract neurons that also respond selectively to histamine (Andrew & Craig, 2001). To date, the electrophysiological work in mice does not indicate whether the itch-associated responses are C-fibre mediated (Maekawa *et al.*, 2000), as described in man and cats. If the work in mice, suggesting a similar itch-specific nerve population to that seen in man and cats, can be further progressed, more could be revealed about the nature of the nerves and receptors which detect pruritogenic stimuli.

Evidence indicating that there are a population of itch specific nerve fibres has not been presented in the literature until recent years. Prior to the studies described above the weight of evidence indicated that the sensations of itch and pain 'shared' the same nerve fibres. Evidence from recordings from human cutaneous nerves showed that pain and itch inducing drugs activated the same fibres (Handwerker *et al.*, 1991). This suggestion was supported by evidence from micro-stimulation of cutaneous C-fibres, in which it was not conclusively shown that it was possible to evoke a sensation of itch, rather than pain in human subjects (Torebjork & Ochoa, 1980). Further development of electrophysiological recordings from cutaneous nerves in mice would provide extra evidence as to whether there is a population of itch-specific nerves.

The aim of the current study was to attempt to develop an *in vivo* model of recording from peripheral afferent nerves innervating the skin at the back of the neck in mice, using electrophysiological techniques, and to use this model to further investigate putative itch evoking compounds, as previously identified from behavioural studies.

8.2 Materials & Methods

The surgical and recording procedures for the neural studies described in this chapter were carried out as described in Chapter 2.3. Experiments in this study were conducted in 36 BalbC mice and 2 ICR mice.

8.3 Results

Spontaneous neural firing of cutaneous afferent nerves

A spontaneous neural signal was identified in 33 of the 38 mice involved in the current study. The reason for the lack of signal in the remaining mice was due to the death of the mice prior to, or during, the surgical procedure. The level of spontaneous firing identified was highly variable. This may have been because of activation of the nerves due to the surgical procedure in some mice. For this reason, most results expressed in this section are based on the duration of the increased firing response and the latency of the peak response, rather than the absolute level of firing induced.

Increased neural firing following the administration of a range of drugs (i.d., see below for details of response to specific compounds) was identified in 13 of the 28 mice, in which drugs were administered.

Peripheral afferent neural responses to histamine

Increased neural firing was evoked in eight of the 24 mice in which histamine (in the range 10-1000 μ g, i.d.) was administered. A typical histamine evoked neural response is shown in figure 8.1. The duration of histamine evoked firing was 833 ± 209 seconds (mean \pm sem, n=11). The latency of peak firing response was 348 ± 118 seconds (mean \pm sem, n=11). Saline did not evoke an increase in action potential firing, as shown in figure 8.2

Histamine did not evoke increased neural firing in afferent nerves innervating the knee joint in mice, unpublished observations from within the laboratory.



Figure 8.1 Typical increase in neural activity following histamine ($1000\mu\text{g}$, i.d.). Upper trace shows impulses per second, lower trace shows raw neural output. Injection indicated by *, also showing injection artifact. Black line indicates 100 seconds. Example of action potential recorded is shown below trace. Neural response peaks at 588 secs and has a duration of 1578 secs.

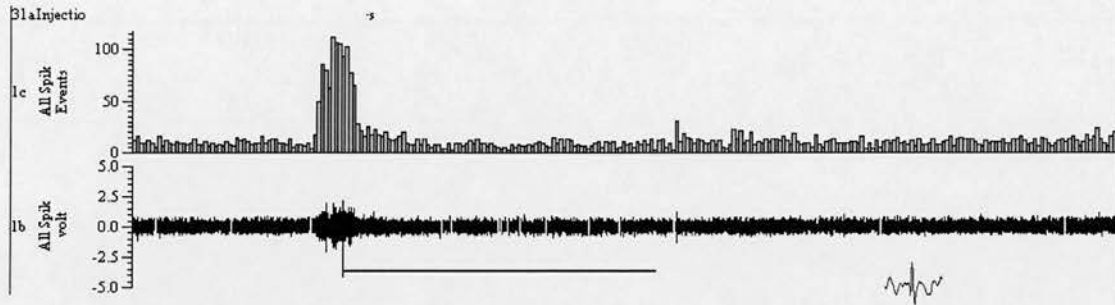


Figure 8.2 Typical neural activity following saline (i.d.). Upper trace shows impulses per second, lower trace shows raw neural output. Injection indicated by 's', also showing injection artifact. Black line indicates 100 seconds. Example of action potential recorded is shown below trace.

Peripheral afferent neural responses to capsaicin

Increased neural firing was evoked in three of the seven mice in which capsaicin (0.3-10 μ g, i.d.) was administered. A typical capsaicin evoked neural response is shown in figure 8.3. The duration of capsaicin evoked firing was 87 ± 21 seconds (mean \pm sem, n=5), significantly lower than that evoked by histamine, described above ($P<0.05$, Mann-Whitney test). The latency of peak firing response to capsaicin was 18 ± 5 seconds (mean \pm sem, n=5), again, significantly less than that evoked by histamine ($P<0.05$, Mann-Whitney test).

Increased neural firing was also identified in one of two mice receiving anandamide (VR1 receptor agonist, 1000 μ g, i.d.). Neural firing in response to anandamide had a latency close in nature to that evoked by capsaicin, 41 seconds (see figure 8.4). The duration of response was, however, greater than we might expect from a typical capsaicin response, 388 seconds, but still lower than that evoked by histamine.

Peripheral afferent neural responses to 5-HT

Increased neural firing was evoked in six of the 11 mice in which 5-HT (1-10 μ g, i.d.) was administered. A typical 5-HT evoked neural response is shown in figure 8.5. The duration of 5-HT evoked firing was 830 ± 162 seconds (mean \pm sem, n=9), not significantly different from that evoked by histamine ($P>0.05$, Mann-Whitney test). The latency of peak firing response to 5-HT was 342 ± 74 seconds (mean \pm sem, n=9), again, not significantly different to that evoked by histamine ($P<0.05$, Mann-Whitney test).

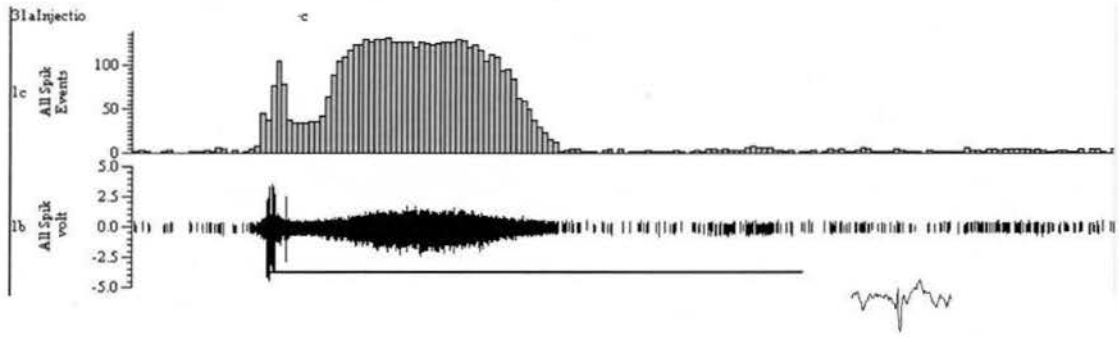


Figure 8.3 Typical increase in neural activity following capsaicin ($0.3\mu\text{g}$, i.d.). Upper trace shows impulses per second, lower trace shows raw neural output. Injection indicated by 'c', also showing injection artifact. Black line indicates 100 seconds. Example of action potential recorded is shown below trace. Neural response peaks at 14 secs and has a duration of 48 secs.

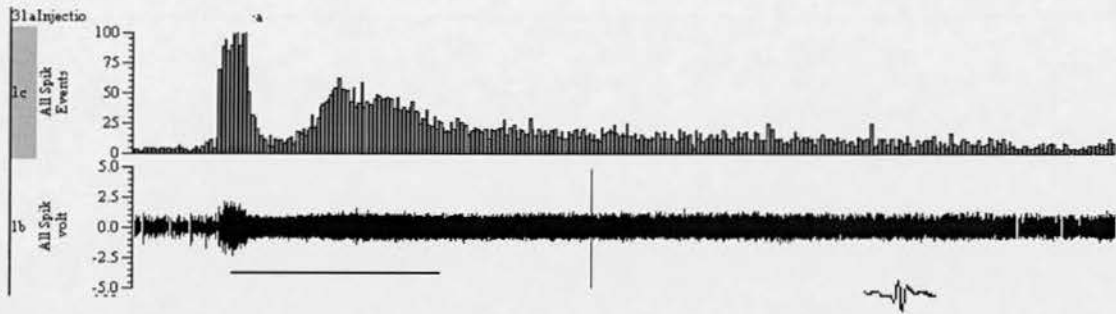


Figure 8.4 Typical increase in neural activity following anandamide (1mg, i.d.). Upper trace shows impulses per second, lower trace shows raw neural output. Injection indicated by 'a', also showing injection artifact. Black line indicates 100 seconds. Example of action potential recorded is shown below trace. Neural response peaks at 41 secs and has a duration of 388 secs.

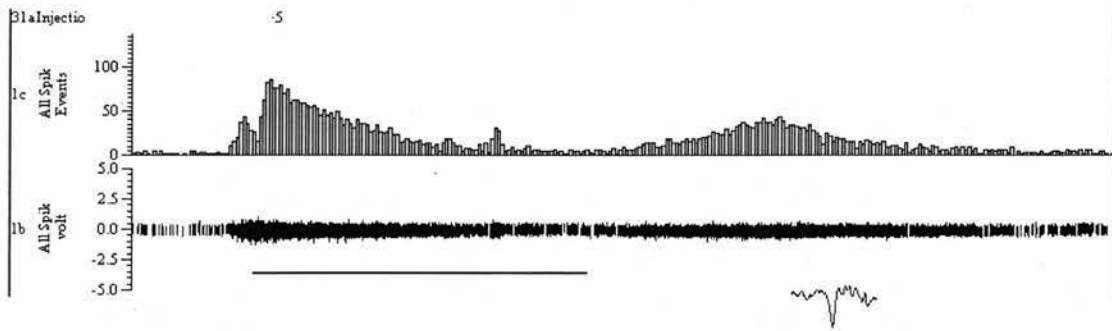


Figure 8.5 Typical increase in neural activity following 5-HT (10 μ g, i.d.). Upper trace shows impulses per second, lower trace shows raw neural output. Injection indicated by '5', also showing injection artifact. Black line indicates 100 seconds. Example of action potential recorded is shown below trace. Neural response peaks at 8 secs and has a duration of 233 secs.

Peripheral afferent neural responses to clobenpropit, histamine H₄ receptor agonist

Increased neural firing was evoked in two of the seven mice in which clobenpropit (3ug, i.d.) was administered. A typical clobenpropit evoked neural response is shown in figure 8.6. The duration of clobenpropit evoked firing was 828 ± 11 seconds (mean \pm sem, n=2), similar to that identified in mice receiving histamine. The latency of peak firing response was 520 ± 292 seconds (mean \pm sem, n=2), again, similar to that observed following histamine.

Afferent nerve units respond to pruritogenic and algogenic stimuli

The five afferent nerve units in which capsaicin (algogenic stimuli) increased neural firing also responded to pruritogenic stimuli. All five of the capsaicin sensitive units also responded positively to 5-HT. Two of the capsaicin sensitive units were also histamine sensitive.



Figure 8.6 Typical increase in neural activity following clobenpropit ($3\mu\text{g}$, i.d.). Upper trace shows impulses per second, lower trace shows raw neural output. Injection indicated by "*", also showing injection artifact. Black line indicates 100 seconds. Example of action potential recorded is shown below trace. Neural response peaks at 228 secs and has a duration of 817 secs.

8.4 Discussion

The studies described within this section of the thesis indicate that it is possible to make *in vivo* electrophysiological recordings from peripheral afferent nerves innervating the skin. This recording technique provides the possibility to investigate itch in mice, without the need for subjective behavioural studies on scratching.

The current study has found that putative pruritogenic drugs, when administered intradermally, evoke increased neural activity in afferent cutaneous nerves. Histamine, 5-HT and clobenpropit (histamine H₄ receptor agonist) all evoked an increase in the number of action potentials recorded. Responses to each drug had a delayed onset, peaking at 6-8 minutes post injection and having a duration of 12-14 minutes. This time course of response is similar to that observed with scratching evoked by these compounds. This evidence indicates that the neural activity evoked is comparable with the scratching behaviour induced in mice, as described in the earlier chapters of this thesis. The findings of the present study are comparable with the existing literature in mice, indicating that histamine and 5-HT induce a time pattern of neural firing, similar to that identified in scratching (Maekawa *et al.*, 2000). Previous work within the laboratory has indicated that in electrophysiological investigations (Gauldie *et al.*, 2001) of rat and mouse nerves innervating the knee joint, histamine evokes no increase in neural activity, unpublished observations. Therefore, the histamine-evoked responses identified from cutaneous nerves suggest that such responses are specifically localised to the skin, as would be expected if itch responses were being investigated.

Capsaicin and anandamide (VR1 receptor agonist) both evoked a pattern of neural response distinct to that induced by 'pruritogenic' drugs. The neural response to both capsaicin and anandamide was a short burst of firing, lasting only seconds of a few minutes, and the response peaked within several seconds. This burst of neural activity is similar to that found in similar recordings made from rat and mouse nerves innervating the knee joint (Gauldie *et al.*, 2001).

The difference in pattern of neural activity evoked by 'algogenic' and 'pruritogenic' compounds indicates that there may be a distinction in the sensory perception mechanisms of the two conditions. Work in humans and cats have indicated that there appear to be independent population of C-fibre nerves that can be described as 'itch specific' (Andrew & Craig, 2001; Schmelz *et al.*, 1997). Findings from the present study in mice have not been able to confirm that there are distinct populations of itch and nociceptive fibres. The current study indicates that although there is a distinct pattern of response to pruritogenic stimuli, the individual units carrying the signal appear to be responsive to both sets of stimulus.

The current investigation indicates that it is possible to study the neural activity of pruritogens in a murine model. Further investigations would be required to determine more about the exact nature of a pruritogenic response in comparison with a nociceptive response. It is plausible to further develop this technique to determine the character profile of the nerve fibres being studied, as work in humans and cats suggests that the sensation of itch in these species is carried by C-fibres (Andrew & Craig, 2001; Schmelz *et al.*, 1997). Studies using other pruritogenic stimuli will be required to determine whether the nature of the response to histamine and 5-HT is identified in response to all pruritogens.

Although the current study has provided an insight into the nature of pruritogenic versus algogenic neural responses, this work was predominantly a series of pilot studies in which to develop cutaneous recording techniques. Various problems were encountered in this study which further development of the techniques described may reduce. The main problem of the current study was the noise encountered in many of the recordings; this meant identifying a drug-evoked response was not possible. Developing an *in vitro* recording technique may reduce much of the noise found using the current *in vivo* procedure, as much of the noise appeared to be due to interference of heartbeat and breathing. Such an approach would also alleviate the need for a more complex anaesthetic and surgical procedure.

In summary, the current study has shown that *in vivo* electrophysiological recordings of cutaneous afferent nerves are possible and that the effects of pruritogenic stimuli on such nerves can be investigated. The distinct pattern of neural response to pruritogenic and algogenic drugs enables further investigation of itch in mice, without the need for subjective behavioural studies. Such an approach opens up a new possibility for the investigation of new mediators of itch, and their mechanism of action in mice.

Chapter 9

General Discussion

9.1 *Acutely-induced scratching in mice*

The primary aim of the studies contained within this thesis were designed to test the hypothesis that the ‘induction of scratching in mice is a useful and robust model of itch, which can be used to provide a guide to pruritic and antipruritic drugs for testing in humans’. The first half of this question was predominantly answered in chapter 3 of this thesis, in which a basic model of acutely induced scratching in mice was developed.

The model developed in this thesis established that scratching could be evoked in mice using intradermal administration of histamine into the back of the neck. This finding supported previous literature that scratching behaviour could be induced and quantified using ‘known pruritogens’ in mice (Inagaki *et al.*, 1999; Kuraishi *et al.*, 1995). The model was developed around the use of mice due to the possibility of using genetically engineered animals in the future, either over or under-expressing receptors or ligands implicated in pilot studies. The development of the model incorporated other possible variables that may have affected the reproducibility of scratching responses. Such variables that were investigated included; strain of mouse, sex of mouse, volume of injected drug and the vehicle in which the drug was administered. The studies described within this thesis should ensure that any scratching evoked could be repeated consistently, within the boundaries of natural biological variability.

The findings of this thesis suggest that scratching in mice is a response to an itchy and not painful stimulus. Some authors have proposed that such animal models of scratching may not directly correlate to the sensation of itch and are therefore flawed when results are interpreted with human clinical conditions in mind (De Castro-Costa *et al.*, 1987; McMahon & Koltzenburg, 1992). However, the findings of this thesis suggest that a significantly lower level of scratching is evoked when a painful stimulus is administered and that the pattern of scratching response is different to that evoked by an itchy stimulus. I believe it is fair to conclude that mice respond by

scratching when presented with an itchy stimulus and that by measuring a scratching response in mice we can be confident of defining the sensation as itch.

Histamine evoked scratching in BalbC mice was investigated in further detail to determine the role of the specific histamine receptor subtypes in itch. The findings of these studies indicate that there is an apparent role for histamine H₁ receptors and also the newly described histamine H₄ receptor. Although there is previous evidence indicating a role for the histamine H₁ receptor in itch in mice (Inagaki *et al.*, 1999; Sugimoto *et al.*, 1998) and man (Greaves & Davies, 1982), the evidence presented within this thesis is the first to implicate a role for the histamine H₄ receptor.

Studies contained within this thesis also illustrated that 5-HT elicited scratching in mice. 5-HT is recognised as a pruritogen in man (Fjellner & Hagermark, 1979) and mice (Andoh & Kuraishi, 1998). The current studies indicated that 5-HT evoked scratching in mice is mediated via the 5-HT₂ and not the 5-HT₃ receptor subtype.

The current studies also investigated the pruritogenic role of PAR-2, previously implicated in itch in man (Steinhoff *et al.*, 2003). The protease, papain, and the PAR-2 specific agonist, SLIGRL, were both shown to evoke scratching in mice.

Future Directions

I believe that the model of acutely induced scratching in mice is a valid and robust method for quantifying the sensation of itch in mice, and as such is a useful basis for determining putative mediators worthy of investigation in human subjects. Although the model itself is reproducible and effective, there are still various areas in which further investigation may be beneficial.

A novel mechanism of histamine induced scratching involving the histamine H₄ receptor has been described within this thesis. However, with the drugs commercially available at the time of research, it is not possible to conclude for certain that histamine evoked scratching is solely histamine H₁ and H₄ receptor mediated. The lack of specificity of some agonists and antagonists leaves some degree of doubt over

these findings, particularly in relation to the role of the histamine H₃ versus H₄ receptor. In the future, investigations using drugs of known activity at each of the receptor subtypes would further clarify the precise role of each of the receptor subtypes.

One of the major aims of this thesis was to attempt to confirm that any novel pruritogenic drugs identified in mice were also pruritogenic in human subjects. The major area of interest from this thesis would be to investigate the role of specific histamine H₄ receptor agonists and antagonists in humans. Unfortunately, due to time constraints and also the lack of commercially available and ethically approved histamine H₄ receptor specific drugs, this was not possible within this thesis. However, I believe that future investigation into the role of histamine H₄ receptors in itch may provide a new technique in the treatment of itch in man.

The murine model of scratching developed would be equally effective at determining the pruritogenic effects of various other drugs administered. The pruritogenic effects of 5-HT and PAR-2 agonists have been investigated within this thesis; however, I believe that further investigation into the receptor mechanism by which these drugs operate may provide novel evidence upon which to develop investigations in human subjects. Further putative mediators not covered by this thesis due to time constraints should also be considered for future investigation. These mediators were described in detail in chapter 1 of this thesis and even the current literature is unlikely to provide a comprehensive description of all the mediators involved in the sensation of itch and new mediators are likely to be described in the literature in the future.

9.2 Automated detection of scratching in mice

One of the specific aims of the studies contained within this thesis was to 'devise and develop an automated mechanism for the detection of scratching in mice'. The studies described within chapter 4 of this thesis explain the development of a scratch detector based on the rhythmical beating characteristic of the hind paw during scratching. This characteristic formed the basis of the only two automated detectors

of scratching in mice, previously described in the literature (Elliott *et al.*, 2000; Inagaki *et al.*, 2003). Both of the models currently existing in the literature involved placing metallic components in or around the hind paw of the mice and placing the mice in cages, surrounded by metallic coils to detect changes in electrical current. Changes in electrical current exhibiting the beating pattern of scratching were identified as scratching. The main purpose of the detector described within this thesis was to identify scratching using a relatively simple and non-invasive procedure. Although the two models described in the literature appear capable of detecting scratching, they involve prior treatment to the mice involved, something that is not necessary with our model. The model developed in these studies is based on the detection of force change in the cage using a sensitive force transducer. Rhythmical changes in force, at a frequency similar to that observed for scratching, are identified as scratching.

As well as detecting the number of bouts of scratching (as can be identified by the human eye), the automated detector can also be used to determine the exact number of scratch movements and the mean number of scratch movements per bout. This capability may be of great benefit if some pruritic drugs were to induce the same number of bouts, but with a greater number of overall scratch movements, than other pruritogens.

The automated detector is currently designed to record four mice at one time but could feasibly be multiplied up to record many more mice at once. The limiting factor in such a set-up would be the number of computers available to record and process the data. Realistically, it is not plausible to visually measure scratching in any more than four mice at any one time, and ideally in no more than two. Therefore, as well as recording additional information, the detector can also significantly reduce the labour intensity associated with the measurement of scratching.

Although I feel confident that the results generated by the automated detector are a true reflection of scratching in mice, the major area of inaccuracy concerns the detection of 'noise' associated with grooming behaviour. Grooming behaviour was

generally identified at a lower frequency than scratching and therefore the detector has built in filters to remove the majority of the background noise. However, despite these precautions, it was still evident that the majority of the detectors inaccuracy was as a result of grooming behaviour.

Future Directions

Further development of the detector is still required in order to gain a more accurate reflection of scratching behaviour in mice. It was never envisaged that the detector would be 100% accurate, however, I believe that further development may produce more accurate results. Despite any inaccuracies of the detector, it has proved to be a very useful tool, capable of saving a large amount of time in the recording of scratching behaviour.

The automated detector may also prove to be of far greater benefit in the detection of chronically induced scratching in mice. To date the detector has only been used for periods of up to twenty minutes. Visual analysis of scratching is feasible over this time-period; however, scratching evoked over a period of hours or days cannot be measured in this way. Visual measurement of such data requires video recording equipment and subsequent analysis, or measuring the mice for only part of the scratching period, leading to a loss of data during times when the mice are not observed. Using the automated detector would allow detection of scratching over longer time periods, the length of which would only be determined by the computer power available for recording.

The automated detector is currently designed to measure repetitive behaviour in mice, of a frequency associated with scratching. Further development would enable the detector to analyse additional repetitive movements (e.g. grooming) or repetitive behaviour in other small animals, such as rats or guinea pigs. Such studies of scratching in other species may provide a greater indication of the pruritogenic effects of the drugs examined in mice. The development of the current model was based on the induction of scratching in mice. This was primarily due to the possibility in the future of genetically engineering mice with up-regulated or knocked

out receptors or ligands, implicated to play a role in scratching. However, further development of genetic engineering in rats, opens up the possibility of such investigations in this species. Therefore, it may prove to be of benefit, in the future, to have an automated detection system capable of operating in both mice and rats.

9.3 Chronically-induced scratching in mice

One of the aims of the studies described within this thesis was to examine the effects of putative mediators of scratching mice. This was primarily achieved by administration of such compounds into the skin of mice, and the subsequent measurement of acutely evoked scratching. This model is suitable for determining the effect of 'itch inducing' drugs but less suitable for examining the effect of 'itch relieving' drugs. The best model for examining itch-relieving drugs is to have a model in which the mice already scratch. One such strain of mice exists where scratching occurs spontaneously (Suto *et al.*, 1999; Vestergaard *et al.*, 2000). The next option is to induce a chronic state of scratching in mice, using drugs, which elicits a long term scratching response.

DNCB was shown in this study to evoke a scratching response in mice over a period of 25 days, when administered topically in a two, three or four dose regimen. Attempts to induce a consistent and chronic state of scratching were not possible using other compounds tested (FCA, papain or the PAR-2 receptor specific agonist papain).

The chronic scratching induced by DNCB was considered significant enough to be able to examine the itch reducing properties of tacrolimus, a recently released itch alleviating drug in man (Cheer & Plosker, 2001). However, Tacrolimus showed no significant reduction in DNCB induced scratching in mice. The scratch reducing effects of histamine H₁ and H₃/H₄ receptor antagonists were also studied, following the results gained in acute scratching studies. Both mepyramine (histamine H₁ receptor antagonist) and thioperamide (histamine H₃/H₄ receptor antagonist) had a

scratch reducing effect on DNCB treated mice. Interestingly, this effect was even more significant when the two antagonists were administered in combination.

Future Directions

The DNCB model used in these studies provides a useful indication of potential itch relieving compounds. However, natural variability in scratching responses between mice did not allow data of statistical significance to be gained in these studies. For this reason one of two possibilities could be implemented in future investigations; more mice could be studied in order to reduce variability, or, mice could be observed for a greater amount of time. In the current studies, mice were observed for two ten-minute periods per day, and it may be possible that for various reasons some mice were not scratching as anticipated at various recording times, for instance, due to tiredness or discomfort. This approach was taken to reduce the labour intensity of the experiment, however, one resolution would be to observe the mice constantly. Using the automated detection system it would be feasible to measure scratching over much longer periods of time. The result of such an approach would be reduce any variation in the times mice do or do not scratch. Now that the automated detector is validated, it would prove beneficial to focus its use on chronic rather than acute scratching studies.

9.4 Electrophysiological studies in mice

The electrophysiological studies contained within this thesis confirm it is possible to record cutaneous afferent nerve activity, and that pruritogenic stimuli evoke an increase in neural activity distinct from that evoked by algogenic stimuli. This finding is comparable to the one other study of itch evoked neural activity in mice (Maekawa *et al.*, 2000). Unlike work in humans (Schmelz *et al.*, 1997) and cats (Andrew & Craig, 2001), the current studies were unable to confirm the presence of a distinct population of itch sensitive nerves. However, although individual units in mice appear to respond to algogenic and pruritogenic stimuli, the pattern of responses are distinct in their time course.

Future Directions

Further refinement of the electrophysiological techniques used in this study would allow a more comprehensive investigation into the character of neural responses to pruritogenic stimuli. The most useful development would be to use an *in vitro* recording system, by removing a patch of skin with the cutaneous nerve attached. Such an approach would remove all efferent neural activity and would also eliminate the predominant source of noise within the system, heartbeat and respiration. This more refined recording technique may allow a greater characterisation of the nerves transmitting the itch signal, specifically; to what stimuli do the nerves respond (e.g., mechano-stimuli, thermo-stimuli). This greater characterisation of the nerves may enable a more directed approach to blocking the itch stimulus, both in murine models and eventually in human clinical itch disorders.

9.5 General conclusions

The results described in this thesis contribute to our overall knowledge concerning scratching, and itch, in mice. The results indicate that this model of scratching in mice provides a sound basis in which to investigate itch, and provides the possibility for novel anti-pruritic compounds in man to be studied. Further to this, an automated detector of scratching in mice has been developed which is capable of gaining more information about the exact nature of the scratching and also significantly reducing the labour intensity otherwise associated with such studies.

A novel mechanism of histamine mediated scratching in mice has been discovered, implicating a role for the recently discovered histamine H₄ receptor in itch. If similar results were to be repeated in human subjects this may open up new possibilities in potential treatments of clinical pruritic disorders in man.

Results also showed that *in vivo* electrophysiological studies from peripheral, cutaneous afferent nerves could be made in mice. Recordings showed that specific patterns of response could be elicited from nerves following administration of pruritic compounds. Such results allow further investigation into the mechanism and

neural pathway of the itch sensation in mice, and of potential new endogenous mediators. Such an approach would allow us to learn more about the sensation of itch in clinical pruritic disorders in man, and may lead to the discovery of novel and more effective antipruritic treatments in the future.

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Appendix I – Drugs & Solutions

Drugs use in this study

Generic Name	Chemical Name	Molecular Weight	Supplier	Solvent
Anandamide	-	347.5	Tocris	PBS
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	1060.2	Sigma	PBS
Capsaicin	8-methyl-N-vanillyl-6-nonenamide	305.4	Sigma	PBS
Cimetidine	-	252.3	Tocris	PBS
Cinanserin	Cinanserin hydrochloride	381.4	Tocris	PBS
Clobenpropit	N-(4-chlorobenzyl-S-[3-4(5)-imidazolyl)propyl]isothiourea dihydrobromide	470.6	Tocris	PBS
Compound 48/80	-	-	Sigma	PBS
Dimaprit	S-(3-dimethylaminopropyl)isothiourea dihydrochloride	234.2	Tocris	PBS
DNCB	1-chloro-2,4-dinitrobenzene	202.6	Sigma	Ethanol
Histamine	Histamine diphosphate	307.1	Sigma	Saline, PBS
HTMT	Histamine-trifluoromethyl-toluidide dimaleate	614.6	Tocris	PBS
5-HT	5-hydroxytryptamine hydrochloride	212.7	Sigma	PBS
Imetit	Imetit dihydrobromide	332.1	Tocris	PBS
Mepyramine	Mepyramine maleate	401.5	Tocris	PBS
Papain	Papain crude	-	Sigma	PBS
SLIGRL	Ser-Leu-Ile-Gly-Arg-Leu-NH ₂	656.82	Tocris	PBS
Substance P	-	1347.6	Sigma	PBS
Terfenadine	-	471.7	Sigma	PBS

Thioperamide	N-cyclohexyl-4-(imidazol-4-yl)- 1-piperidinecarbothioamide maleate	408.5	Tocris	PBS
3, tropanylindole 3, carboxylate methiodide	-	426.3	Sigma	PBS
Trypsin	(from porcine pancreas) 1:250	Activity: 1120 BAEE units/mg	Sigma	PBS

Solutions

Saline: 0.9g NaCl in 100ml distilled water

PBS: 40.5 ml 0.2M Na₂HPO₄. 12H₂O in saline
9.5ml 0.2M Na₂H₂PO₄. 2H₂O in saline
50ml saline

Appendix II – Publications

Involvement of histamine H₄ and H₁ receptors in scratching induced by histamine receptor agonists in BalbC mice

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1 The role of histamine H₁, H₂, H₃ and H₄ receptors in acute itch induced by histamine was investigated in female BalbC mice. Scratching was induced by intradermal injections of pruritogen into the back of the neck and 'itch' assessed by quantifying the scratching evoked.

2 Histamine (0.03–80 µmol), histamine-trifluoromethyl-toluidine (HTMT, H₁ agonist, 0.002–2 µmol), clobenpropit (H₄ agonist, H₃ antagonist, 0.002–0.6 µmol) and to a lesser extent imetit (H₃/H₄ agonist, 0.03–3 µmol) all induced dose-dependent scratching. Dimaprit (H₂ agonist, 0.04–40 µmol) did not cause scratching.

3 Mepyramine (H₁ antagonist, 20 mg kg⁻¹, i.p.) reduced scratching evoked by histamine and HTMT, but not that caused by H₃ or H₄ agonists. Thioperamide (H₃/H₄ antagonist, 20 mg kg⁻¹, i.p.) reduced scratching induced by histamine, H₃ and H₄ agonists, but not that caused by HTMT. The non-sedating H₁ antagonist, terfenadine, also significantly reduced the scratching induced by the H₁ agonist, HTMT. Cimetidine (H₂ antagonist, 20 mg kg⁻¹, i.p.) did not affect histamine-induced scratching.

4 These results indicate that activation of histamine H₄ receptors causes itch in mice, in addition to the previously recognised role for H₁ receptors in evoking itch. Histamine H₄ receptor antagonists therefore merit investigation as antipruritic agents.

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Keywords: Itch; pruritus; histamine; mouse; H₁ receptor; H₃ receptor; H₄ receptor

Abbreviations: HTMT, histamine-trifluoromethyl-toluidine; H₁, histamine H1 receptor; H₂, histamine H2 receptor; H₃, histamine H3 receptor; H₄, histamine H4 receptor; i.d., intradermal; i.p., intraperitoneal; PBS, phosphate buffered saline

Introduction

Itch (pruritus) is commonly defined in humans as an unpleasant sensation of the superficial layers of the skin (Shelley & Arthur, 1957) provoking the desire to scratch (Ekblom, 1995). It is a common clinical condition that can be associated with cutaneous (e.g. atopic eczema, contact dermatitis) or systemic (e.g. chronic renal failure) disease. Itch is difficult to study objectively in man and there are currently few reliable animal models of itch.

Histamine has long been recognised as a mediator of itch in humans (Lewis, 1927). Although traditional H₁ receptor antagonists are effective at reducing histamine-induced itch (Hagermark *et al.*, 1979) and are widely used clinically for treating pruritus (Greaves, 1997), they are not effective antipruritics in many clinical conditions, such as atopic eczema (Yosipovitch *et al.*, 2003). It has been claimed that the effectiveness of H₁ receptor antagonists in relieving itch results from central sedation rather than peripheral actions on sensory nerves, in conditions where histamine is not considered to be the primary mediator (Savin, 1980; Krause & Shuster, 1983; Savin *et al.*, 1986). Histamine H₂ receptor antagonists are also used clinically for treating itch (Monroe *et al.*, 1981; Ring *et al.*, 1999), but their ability to inhibit itching is not conclusive (Hagermark *et al.*, 1979; Davies & Greaves, 1980). Overall the

evidence suggests that mechanisms other than those involving H₁/H₂ receptors are involved in itch.

In murine studies, itch is estimated by measuring the scratching elicited by itch-provoking agents (Kuraishi *et al.*, 1995). Although some reports have questioned the role of histamine as a pruritogen in mice (Kuraishi *et al.*, 1995), others have shown that histamine H₁ receptor antagonists are effective at reducing histamine-induced scratching (Sugimoto *et al.*, 1998) in this species. In studies involving mice, it is necessary to appreciate that differences in strain can markedly influence scratching responses to drugs (Inagaki *et al.*, 2001). Concerns have been raised that scratching in animal models of itch may reflect pain rather than pruritus (McMahon & Koltzenburg, 1992), but evidence shows that pain and itch do promote different behavioural responses in mice (Kuraishi *et al.*, 1995; Laidlaw *et al.*, 2002).

The present study was undertaken to investigate the role of H₁, H₂, H₃ and H₄ receptors in histamine-induced itch in BalbC mice based on the quantification of acutely induced scratching.

Methods

Animals

All experiments were performed under U.K. Home Office regulations. Female BalbC mice (Charles River, Margate,

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Kent, U.K.), 10-weeks old, were used in the experiments. Mice weighed 18–26 g and were housed under controlled light (07:00–19:00 h) and temperature (22°C) with food and water available *ad libitum*. Each mouse was used for only one experiment.

Materials

Histamine diphosphate (Sigma, Poole, U.K. MW = 307) was dissolved in saline (0.9% w v⁻¹ aqueous NaCl solution). Histamine–trifluoromethyl–toluidine dimaleate (HTMT; H₁ receptor agonist, MW = 615), dimaprit dihydrochloride (H₂ agonist, MW = 234), imetit dihydrobromide (dual H₃ and H₄ agonist, MW = 332), clobenpropit dihydrobromide (H₄ agonist and H₃ antagonist, MW = 471), mepyramine maleate (sedating H₁ antagonist, MW = 401), terfenadine (nonsedating H₁ antagonist, MW = 472), cimetidine (H₂ antagonist, MW = 252) and thioperamide maleate (H₃ and H₄ antagonist, MW = 413) (all Tocris, Avonmouth, U.K. unless specified) were dissolved in phosphate-buffered saline (PBS, pH = 7.4). All drugs were injected in a volume of 100 µl.

Procedure

The itch-inducing properties of histamine and H₁, H₂, H₃ and H₄ agonists were studied by administering the drugs intradermally (i.d.) into the back of the neck *via* a 26 G needle. Drugs were administered at 1-h intervals. Injection of PBS was used as a control. The injection site was chosen as it is only accessible by the animal's hind paws and therefore scratching behaviour can be separately identified from grooming, which is performed by the forelimbs.

In experiments involving antagonists, scratching was induced by a mid-range dose of histamine, which was repeated 30 and again 90 min after treatment with either an H₁, H₂ or H₃ antagonist (20 mg kg⁻¹, i.p.). This dose of antagonist was selected on the basis of its effectiveness in reducing scratching induced by H₁, H₂ or H₃ antagonists.

The effects of antagonists on receptor-selective agonist-induced scratching were studied in separate groups of mice, as desensitisation to the agonists precluded repeated dosing. The response to agonist was recorded 30 min after antagonist treatment and compared to responses obtained from vehicle (saline)-treated mice. The dose of agonist was selected on the basis of its ability to elicit scratching in each mouse studied, and with the aim of eliciting approximately 50% of maximum scratching response.

Measurement of itch

Mice were filmed with a video camera (Vista, NCD 132) and the signal recorded on a VCR (Panasonic, NV-HD640). 'Itch' was measured by counting the number of 'bouts' of scratching in the 20-min period immediately following injection of histamine or selective H₁, H₂, H₃ or H₄ agonist. A bout of scratching was defined as three or more individual rapid scratch movements with the hind paws to the area around the injection site (i.e. the back of the neck).

Statistical analysis

All data were analysed using GraphPad Prism (v3.02) software. Log dose–response curves were plotted using the

nonlinear regression (sigmoidal dose–response) function. The mean 'apparent' ED₅₀ was calculated from the pooled data and was the dose required to elicit half the apparent maximal response. Nonparametric statistical analysis was used and the null hypothesis was rejected at $P < 0.05$.

The effects of antagonists on histamine-induced scratching were analysed using the Wilcoxon matched pairs test, comparing the response to a dose of histamine preantagonist with the response to the same dose 30 min after antagonist. A second postantagonist response to the dose of histamine was also recorded 90 min after antagonist, to give an indication of the antagonist's duration. The effect of antagonist on agonist-induced scratching was statistically analysed using the Mann–Whitney test.

Results

Histamine-induced scratching

Histamine evoked dose-dependent scratching in all the mice studied, as summarised in Figure 1. Scratching was not obtained at lower doses (up to 0.8 µmol), but was consistently observed with higher doses, reaching a maximum at 26 µmol histamine. Above this dose there was no further increase, and often a decrease, in scratching. Histamine-induced scratching reached a maximum of 73 ± 12 bouts (mean ± s.e.m.), with a mean apparent ED₅₀ of 5.8 µmol.

Scratching induced by phosphate-buffered saline served as a control (5 ± 1 bouts of scratching, $n = 12$).

A painful stimulus (9 M HCl, 0.1 ml, i.d. $n = 4$) did not induce scratching (0 ± 0 bouts) and was associated with behaviour not seen with histamine (vocalisation upon injection and 'hunching' after the injection).

Role of histamine H₁ receptors in histamine agonist-induced scratching

The selective histamine H₁ receptor agonist, HTMT, evoked dose-dependent scratching in all six mice studied (Figure 2a). Maximal scratching was 133 ± 17 bouts, with a mean apparent ED₅₀ for HTMT of 0.1 µmol.

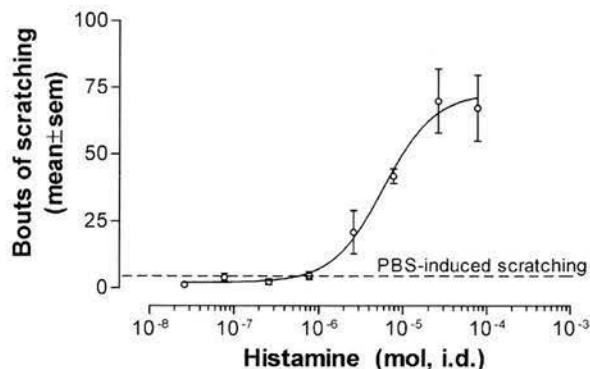


Figure 1 Pooled data for histamine-induced scratching in female BalbC mice during the 20-min postinjection period ($n = 4-8$). Histamine caused dose-dependent scratching. Dashed line represents the mean level of scratching induced by PBS, which served as a control (5 ± 1 bouts of scratching, $n = 12$).

Histamine-induced scratching (8 μmol , i.d.) was significantly reduced following mepyramine (20 mg kg^{-1} i.p.; $P < 0.05$, $n = 6$, Figure 3a). Saline (0.9% w/v aqueous NaCl solution), used as a control for the antagonist, did not significantly reduce histamine-induced scratching ($P > 0.05$, $n = 5$, Figure 3b).

HTMT-induced scratching (0.2 μmol i.d.) was significantly reduced in mice pretreated with mepyramine (20 mg kg^{-1} i.p.) in comparison with vehicle-treated mice ($P < 0.05$, Figure 3c). Lower doses of mepyramine (1–10 mg kg^{-1}) did not significantly reduce HTMT-induced scratching.

HTMT-induced scratching (0.2 μmol i.d.) was also significantly reduced by pretreatment with the non-sedating histamine H_1 receptor antagonist, terfenadine (20 mg kg^{-1} i.p.) as compared with vehicle-treated mice ($P < 0.05$, Figure 3d). Lower doses of terfenadine (1–10 mg kg^{-1}) did not significantly reduce HTMT-induced scratching ($P > 0.05$) but there was variability with responses, and the trend indicated that the antiscratch effect of terfenadine was dose related.

Role of histamine H_2 receptors in histamine agonist-induced scratching

Dimaprit, a histamine H_2 receptor agonist, did not induce dose-dependent scratching in mice (0.04–40 μmol , $n = 3$, Figure 2d). The H_2 antagonist cimetidine (20 mg kg^{-1} i.p.) did not significantly reduce histamine-induced scratching ($P > 0.05$, $n = 6$; data not shown).

Role of histamine H_3 receptors in histamine agonist-induced scratching

Imetit, a histamine H_3 and H_4 receptor agonist, induced dose-dependent scratching in all six mice studied (Figure 2c). The maximal scratching was 69 ± 23 bouts, with a mean apparent ED_{50} for imetit of 0.9 μmol .

Scratching induced by histamine (3 μmol i.d.) was significantly reduced after treatment with the H_3/H_4 antagonist thioperamide (20 mg kg^{-1} i.p.; $P < 0.05$, $n = 6$, Figure 4a). Saline vehicle (i.p.) did not significantly reduce histamine-induced scratching ($P > 0.05$, $n = 6$, Figure 4b).

Imetit-induced scratching (3 μmol i.d.) was significantly reduced in mice pretreated with thioperamide (20 mg kg^{-1} i.p.) in comparison with vehicle-treated animals ($P < 0.05$, Figure 4c). Lower doses of thioperamide (1–10 mg kg^{-1}) reduce the mean level of imetit-induced scratching, but due to variability in responses, the difference between means (*versus* saline) was not statistically significant ($P > 0.05$) (Figure 5).

Role of histamine H_4 receptors in histamine agonist-induced scratching

Clobenpropit, an H_4 agonist and H_3 antagonist, induced dose-dependent scratching in all 10 mice studied (Figure 2b). The maximal level of scratching was 144 ± 20 bouts, with a mean apparent ED_{50} for clobenpropit of 0.05 μmol .

Clobenpropit-induced scratching (0.06 μmol , i.d.) was significantly lower in mice pretreated with the highest doses of thioperamide used (10 and 20 mg kg^{-1} , i.p.) when compared to vehicle-treated mice ($P < 0.05$, Figure 6).

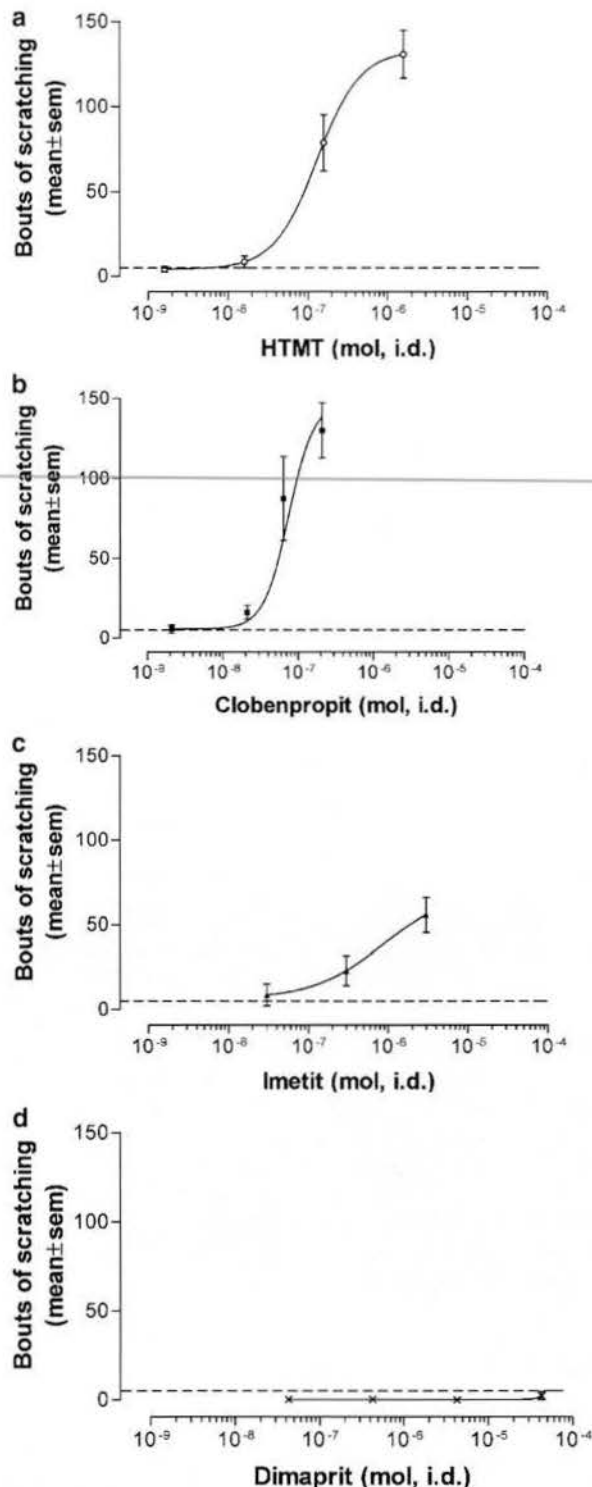


Figure 2 Pooled data illustrating scratching induced by (a) HTMT (H_1 receptor agonist, $n = 6$), (b) clobenpropit (H_4 agonist, H_3 antagonist, $n = 10$), (c) imetit (H_3/H_4 agonist, $n = 6$) and (d) dimaprit (H_2 agonist, $n = 3$) during the 20 min postinjection. HTMT, clobenpropit, and to a lesser extent imetit, caused dose-related scratching, whereas dimaprit had no effect (dashed line illustrates PBS-induced scratching).

H_1 versus H_3/H_4 receptor involvement in itch

The H_1 agonist, HTMT (0.2 μmol , i.d.), induced scratching that was not significantly reduced by the H_3/H_4 receptor

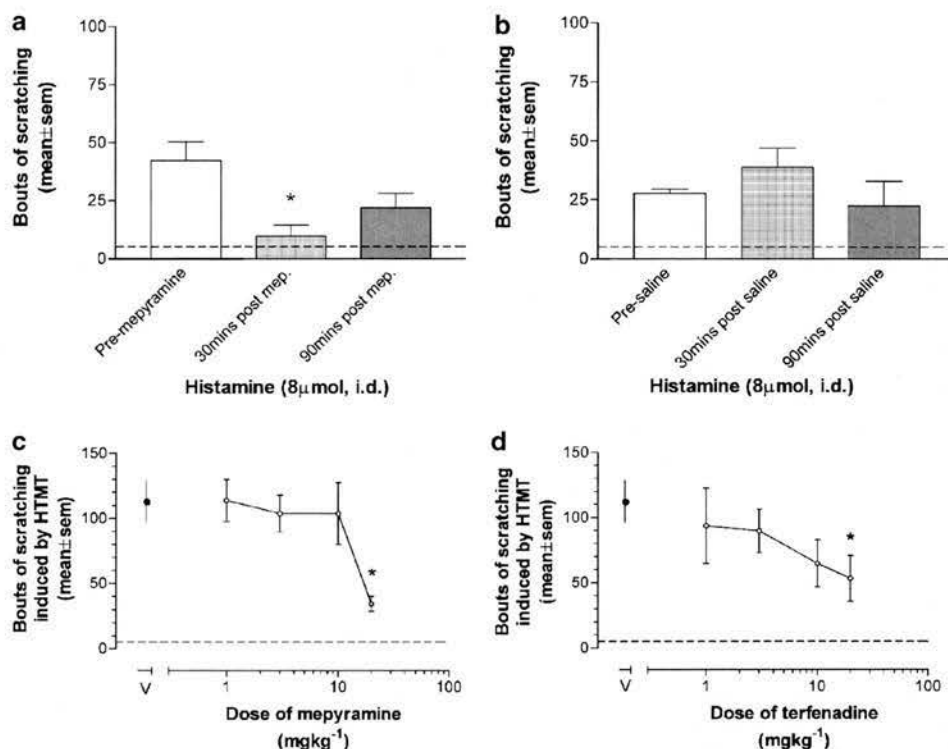


Figure 3 (a) Mepyramine (20 mg kg⁻¹ i.p.) significantly reduced scratching induced by histamine (8 μmol, i.d.) in female BalbC mice, 30 min after treatment with the antagonist *versus* pretreatment value (**P* < 0.05, Wilcoxon matched pairs test, *n* = 6). (b) Saline vehicle did not significantly reduce scratching induced by histamine (*P* > 0.05, Wilcoxon matched pairs test, *n* = 5). (c) Mepyramine (20 mg kg⁻¹ i.p.) pretreated mice showed a significantly lower scratching response to HTMT (0.2 μmol i.d.) in comparison with vehicle-treated animals (**P* < 0.05, Mann–Whitney test, *n* = 6 in each group). Mepyramine at lower doses (1, 3 or 10 mg kg⁻¹) did not significantly reduce HTMT-induced scratching (*P* > 0.05, Mann–Whitney test, *n* = 6 in each group). (d) Terfenadine significantly reduced scratching induced by HTMT (0.2 μmol i.d.) in a dose-dependent manner (terfenadine 20 mg kg⁻¹ *versus* control, **P* < 0.05, Mann–Whitney test, *n* = 6 in each group) (V = vehicle for antagonist. Dashed line illustrates PBS-induced scratching).

antagonist, thioperamide (20 mg kg⁻¹, Figure 6a, *P* > 0.05 *versus* vehicle), whereas it was by the H₁ receptor antagonists, terfenadine and mepyramine (as described above).

The H₄ agonist, clobenpropit (0.06 μmol i.d.), induced scratching that was not significantly reduced by either mepyramine (sedating), or terfenadine (nonsedating) H₁ receptor antagonists (20 mg kg⁻¹, Figure 6b, *P* > 0.05 *versus* vehicle), whereas it was by the H₃/H₄ receptor antagonist thioperamide (see above).

Discussion

Results from this study indicate the involvement of histamine H₄ receptors in the scratching (itch) evoked by histamine, in mice. The lack of effectiveness of classical H₁ receptor antihistamines in alleviating many chronic pruritic conditions (Greaves, 1997) led to the focus of research for antipruritic drugs being shifted from histamine to other putative mediators. The present study suggests that histamine can generate itching in mice *via* H₄ and possibly H₃ receptors.

We confirmed that acutely administered histamine induces dose-dependent scratching when injected intradermally in BalbC mice (Inagaki *et al.*, 2001), as it does in humans (Keele & Armstrong, 1964). It has previously been shown in mice that the dose of histamine required to induce scratching behaviour in this species is substantially higher (approximately 100 times)

than that which evokes itch in humans (Kuraishi *et al.*, 1995; Maekawa *et al.*, 2000; Inagaki *et al.*, 2001). This probably reflects species differences in the properties of histamine receptors, as has been shown to be the case for mice and man (Liu *et al.*, 2001), but caution is needed when interpreting results from functional studies undertaken in mouse or man. The H₁ antagonist, mepyramine, reduced histamine-induced scratching, as previously established in mice (Sugimoto *et al.*, 1998) and humans (Hagermark *et al.*, 1979). Some authors have suggested this may, at least in part, be due to the sedating properties of H₁ antagonists rather than a direct peripheral action at pruritoceptors (Krause & Shuster, 1983). We have shown that i.d. injection of the histamine H₁ receptor agonist HTMT induces acute scratching in mice, strongly suggesting the involvement of peripheral H₁ receptors in itch. This action of HTMT was mediated *via* H₁ receptors because mepyramine reduced agonist induced scratching. We also showed that a nonsedating H₁ receptor antagonist, terfenadine, significantly reduced HTMT-induced scratching, thus suggesting that sedation is not responsible for the reduction in scratching observed following H₁ receptor antagonists.

Further studies investigating the mechanism of histamine-induced scratching in other strains of mouse and in other species, including humans, would be desirable. In preliminary studies using ICR mice, we found that they were more sensitive by a factor of approximately 30 to histamine, in comparison with BalbC mice. Variability in individual responses meant

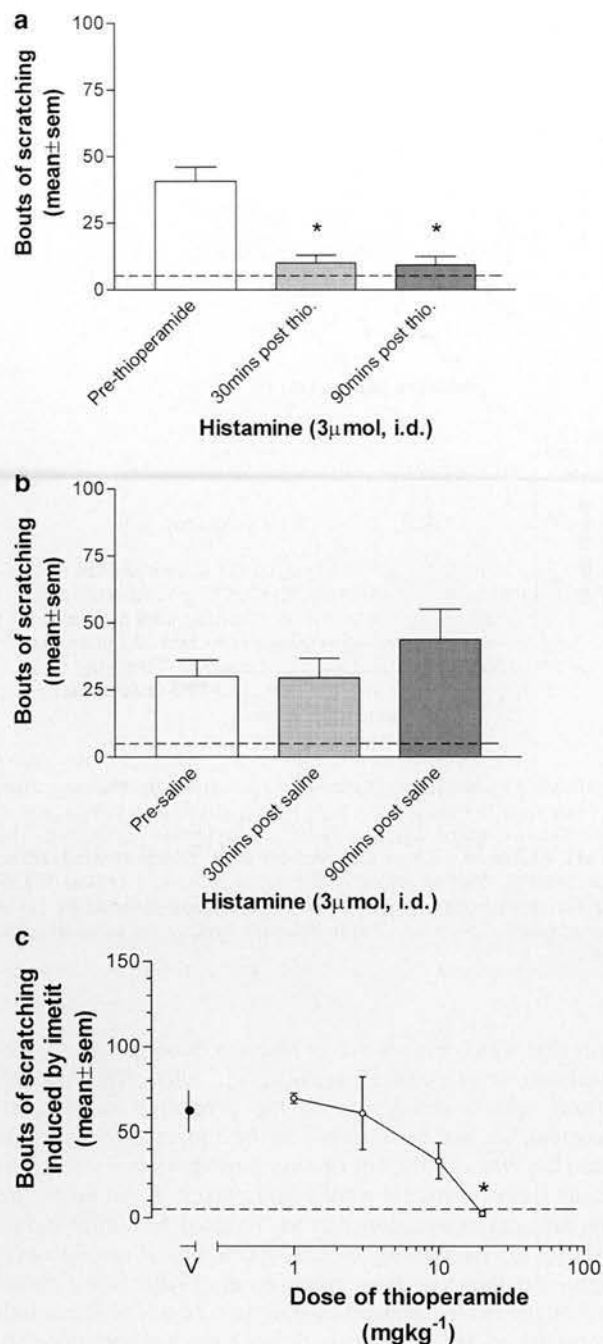


Figure 4 (a) Thioperamide (20 mg kg⁻¹ i.p.) significantly reduced scratching induced by histamine (3 μmol, * $P < 0.05$, Wilcoxon matched pairs test, $n = 6$). (b) Saline vehicle did not significantly reduce scratching induced by histamine ($P > 0.05$, Wilcoxon matched pairs test, $n = 6$). (c) Thioperamide (20 mg kg⁻¹ i.p.) pretreated mice showed a significantly lower scratching response to imetit (3 μmol, i.d.) in comparison with vehicle treated animals (* $P < 0.05$, Mann-Whitney test, $n = 5$ in each group). Thioperamide at lower doses (1, 3 or 10 mg kg⁻¹) did not significantly reduce imetit-induced scratching ($P > 0.05$, Mann-Whitney test, $n = 4$ in each group) (V = vehicle for antagonist. Dashed line illustrates PBS-induced scratching).

that we were unable to obtain clear evidence from the ICR strain concerning the effects of agonists and antagonists selective for different histamine receptor subtypes.

We found no evidence for involvement of H₂ receptors in the scratching evoked by histamine in the mice studied. The H₂

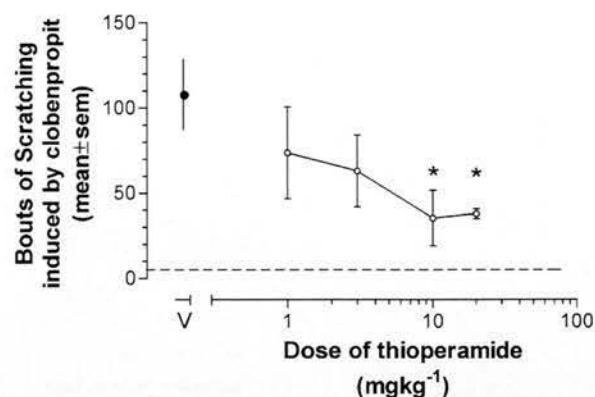


Figure 5 Thioperamide (20 and 10 mg kg⁻¹ i.p.) pretreated mice showed a significantly lower scratching response to clobenpropit (0.06 μmol, i.d.) in comparison with vehicle-treated animals (* $P < 0.05$, Mann-Whitney test, $n = 6$ in each group). Thioperamide at lower doses (1, or 3 mg kg⁻¹) did not significantly reduce imetit-induced scratching ($P > 0.05$, Mann-Whitney test, $n = 6$ in each group) (V = vehicle for antagonist. Dashed line illustrates PBS-induced scratching).

receptor agonist, dimaprit, did not evoke scratching, and the H₂ receptor antagonist, cimetidine, had no significant effect on histamine-induced scratching. This is consistent with evidence in the literature: although a combination of H₁ and H₂ histamine antagonists is sometimes used clinically for treating itch (Drake *et al.*, 1994; Ring *et al.*, 1999), H₂ receptors are not thought to be crucial in histamine-evoked itch (Hagermark *et al.*, 1979; Davies & Greaves, 1980).

The discovery of histamine H₃ (Haaksma *et al.*, 1990) and, more recently, H₄ (Oda *et al.*, 2000) receptors, has implications for histamine as a mediator of itch (Repka-Ramirez, 2003). The tissue distribution of both receptor subtypes indicates a potential role for them in the periphery: the H₃ receptor affects histamine-mediated axonal transport in mouse dorsal root ganglia (Amano *et al.*, 2001), whereas the H₄ receptor is associated with immune responses, is preferentially expressed on leukocytes (Oda *et al.*, 2000), and plays a role in mast cell chemotaxis (Hofstra *et al.*, 2003). We found that the dual H₃ and H₄ receptor antagonist thioperamide significantly reduced histamine-induced scratching in mice. The nonselective H₃ and H₄ receptor agonist, imetit, evoked scratching that was reduced by thioperamide. In order to differentiate between histamine H₃ and H₄ mechanisms, we used clobenpropit, which is an H₃ antagonist but an H₄ agonist (Oda *et al.*, 2000). Clobenpropit induced dose-dependent scratching in mice, which was antagonised by thioperamide, a finding that implicates a peripheral H₄ receptor in scratching induced by i.d. histamine.

We conclude that distinct H₁ and H₄ receptor mechanisms are responsible for itching evoked by exogenous histamine, and this is supported by data showing that scratching induced by H₁ agonist was not significantly reduced by the H₃/H₄ antagonist. Also, scratching evoked by an H₄ agonist was not significantly reduced by sedating or nonsedating H₁ receptor antagonists.

Although our data strongly suggests a role for H₁ and H₄ receptors in histamine-mediated scratching, we were unable to determine whether H₃ receptors play a role. This is because imetit, thioperamide and clobenpropit act at multiple histamine receptor subtypes. The H₃/H₄ receptor agonist imetit induced less scratching than the H₁ and H₄ agonists, which could be because imetit has a low affinity for the murine H₄

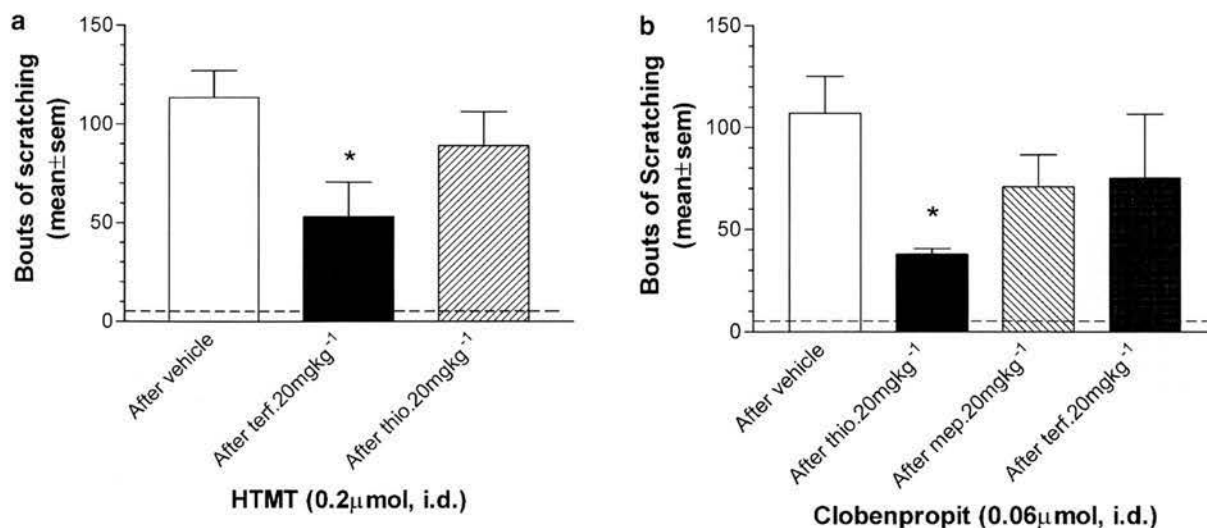


Figure 6 (a) Thioperamide (20 mg kg⁻¹ i.p.) did not significantly reduce HTMT-induced scratching (0.2 μmol i.d.) in comparison to vehicle treated mice ($P > 0.05$, Mann-Whitney test, $n = 6$ in each group), whereas the H₁ antagonist, terfenadine (20 mg kg⁻¹ i.p.) did reduce HTMT-induced scratching ($*P < 0.05$, Mann-Whitney test, $n = 6$ in each group). (b) Mepyramine and terfenadine, sedating and nonsedating H₁ receptor antagonists, respectively (20 mg kg⁻¹ i.p.) did not significantly reduce clobenpropit-induced scratching (0.06 μmol i.d.) in comparison to vehicle-treated mice ($P > 0.05$, Mann-Whitney test, $n = 6$ in each group), whereas the H_{3/4} antagonist, thioperamide (20 mg kg⁻¹ i.p.) did reduce clobenpropit-induced scratching ($*P < 0.05$, Mann-Whitney test, $n = 6$ in each group). Dashed line illustrates PBS-induced scratching.

receptor, or it may be due to a combined H₃ and H₄ action. It has recently been reported that mepyramine binds to the H₄ receptor; which may explain why H₁ antihistamines can relieve itch in some clinical conditions, even if H₁ receptors are not involved (Nguyen *et al.*, 2001). Further studies using drugs with greater selectivity for histamine receptor subtypes, particularly selective H₄ agonists and antagonists are required to distinguish between an H₃ and/or an H₄ mechanism, but based on the use of currently available drugs, we conclude that H₄ as well as H₁ receptors contribute to itch evoked by histamine in mice.

We cannot be certain that the responses to histamine receptor agonists that we observed result from direct actions

on pruritoceptors (the most likely explanation), or indirect actions *via* mast cells, basophils, etc. (Ring & Thomas, 1989; Poli *et al.*, 1994); electrophysiological studies on pruritoceptor afferents in mice and humans would help clarify this. Nevertheless, we believe the results from our functional studies provide sufficient evidence to warrant further studies on the involvement of H₄ receptors in the physiology and pathophysiology of itch in both mouse and man.

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